Gene Regulation and Signal Transduction in *Arabidopsis thaliana*:

1. Regulation of Chlorophyll Biosynthesis at the Level of Glutamyl-tRNA Reductase (*HEMA1*)
2. Signal Transduction via the Protein Phosphatase 2A (PP2A) Pathway

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For all of you who would not let me give up.

Thank you.

Table of Contents

1.1. Abstract

The first common precursor molecule for chlorophyll and heme biosynthesis, 5-aminolevulinic acid (ALA), is formed in chloroplasts of *Arabidopsis* via the C_5-pathway from tRNA-bound glutamate. In this thesis, the light-mediated regulation of the *A. thaliana HEMA1* gene encoding the initial enzyme of the pathway, glutamyl-tRNA reductase (GluTR), was investigated. Light-dependent *HEMA1* mRNA accumulation in cotelydons of *Arabidopsis* seedlings was observed. Promoter deletion mutants fused to the reporter gene β-glucuronidase (GUS) were tested for transient expression in protoplasts as well as in stable transgenic plants. The influence of light and dark treatments as well as the influence of light of different wavelengths was measured. A promoter region 451 bp upstream of the translational start codon including various transcriptional start points was shown to be sufficient in mediating positive light induction. This light response was seen towards blue and red light. The possible participation of the phytochrome system was concluded. A second promoter region was found to be responsible for *HEMA1* gene repression in the dark. This process was found to be dependent on protein phosphatase 1/2A. Finally, a silencing promoter part was discovered. This initial elucidation of the complex *HEMA1* promoter structure reflects the central need for a tightly regulated tetrapyrrole biosynthesis in plants.

Reversible protein phosphorylation is an essential regulatory mechanism in many cellular processes. In a second part of this thesis, the so far poorly understood regulatory role of protein phosphatase 2A (PP2A) in plants was investigated using a two-hybrid screen and a PCR-based screen of a T-DNA mutant collection of *Arabidopsis*. A total of 32 potential interaction partners for two of the catalytic subunits of PP2A in *Arabidopsis* were identified. Surprisingly, multiple proteins of signal transduction, transcriptional regulation and various stress-adaptive processes including osmolarity regulation and infection response were found. Furthermore, processes of protein production, folding and degradation seem to be under partial PP2A control. Finally, indications for a direct influence of PP2A on the control of metabolic pathways and the energy generating machinery were discovered. These observations identify protein phosphatase 2A as a central molecular switch in plants involved in the coordination of important adaptive and developmental processes.

1.2 Zusammenfassung

Das erste gemeinsame Vorläufermolekül für die Chlorophyll- und Hämbiosynthesis, 5-Aminolevulinsäure (ALA), wird in den Chloroplasten von *Arabidopsis* über den C_5-Weg aus tRNA-gebundenem Glutamat geformt. In dieser Arbeit wurde die Lichtregulation des *HEMA1* Genes von *A. thaliana*, das für das erste Enzym dieses Syntheseweges Glutamyl-tRNA Reduktase kodiert, untersucht. Die lichtabhängige Anhäufung von *HEMA1* mRNA in Keimblättern (Cotelydonen) von *Arabidopsis* Keimlingen konnte beobachtet werden. Promotor-Deletionsmutanten, die mit dem bakteriellen Reportergen β-Glucuronidase (GUS) fusioniert waren, wurden sowohl mittels transienter Expressionsversuche in Protoplasten als auch in stabilen transgenen Pflanzen getestet. Der Einfluß von Licht und Dunkelheit sowie von Licht verschiedener Wellenlängen auf die Reportergenexpression wurde gemessen. Eine Promotorregion 451 bp stromaufwärts vom Translations-Startcodon, die auch verschiedene Transkriptionstartpunkte beinhalted, war ausreichend, um eine positive Lichtinduktion zu vermitteln. Diese Lichtantwort wurde sowohl mit blauem als auch mit roten Licht beobachtet. Eine mögliche Beteiligung des Phytochromsystems an der Lichtantwort wurde gefolgert. Eine zweite Promotorregion, die für die Repression der *HEMA1* Genexpression in Dunkelheit verantwortlich ist, wurde identifiziert. Es konnte gezeigt werden, daß dieser Prozeß von Protein-Phosphatasen 1/2A abhängig ist. Schließlich konnte ein Promotorteil mit *Silencing*-Funktion identifiziert werden. Diese ersten experimentellen Hinweise auf eine komplexe *HEMA1*-Regulation spiegeln die zentrale Bedeutung einer strikten Steuerung der Tetrapyrrolbiosynthese in Pflanzen wieder.

Reversible Proteinphosphorylation ist ein zentrales Regulationsprinzip vieler zellulärer Prozesse. In einem zweiten Teil dieser Arbeit wurde die für Pflanzen schlecht verstandene regulatorische Rolle der Protein-Phosphatase 2A (PP2A) über die Durchführung eines *Two-Hybrid Screens* und mittels Durchmusterung einer T-DNA Mutantensammlung auf PCR-Basis untersucht. Insgesamt konnten 32 potentielle Wechselwirkungspartner für zwei der katalytischen Untereinheiten der PP2A identifiziert werden. Überraschenderweise wurden mehrere Proteine der pflanzlichen Signaltransduktion, transkriptionellen Regulation und verschiedener Prozesse der Stressadaption, wie Osmolaritätsregulation und Infektionsantwort gefunden. Auch die Proteinproduktion, Proteinfaltung und der Abbau von Protein könnten teilweise unter der Kontrolle von PP2A stehen. Schließlich wurden Anzeichen für einen direkten Einfluß der PP2A auf Steuerung des Stoffwechsels und des Energiehaushalts entdeckt. Diese Beobachtungen identifizieren Protein-Phosphatase 2A als zentralen molekularen Schalter in Pflanzen, der an der Koordination grundlegender adaptiver Prozesse der pflanzlichen Entwicklung und des Überlebens beteiligt ist.

2. Introduction

2.1. Plants and Environment

A plant is exposed to a multitude of abiotic and biotic environmental factors that vary both spatially and temporally. Biotic factors may include competitors, herbivores, diseases of various sorts including virus infections and pathogenic fungi as well as parasites. Among abiotic influences may be variation in temperature (cold or heat shock), availability of nutrients and water (drought, salinity levels, pH levels), mechanical stress (touch, wind) and the quality and direction of light. As sessile organisms plants are unable to move away from unfavorable environmental conditions. Therefore, it is important that they are able to respond rapidly to changes in environmental signals to improve their chances of survival.

Examples for plant responses to environmental stimuli are phototropism and gravitropism that describe the plant's orientation according to light and the gravity vector, respectively. In *Arabidopsis*, phototropism is manifested by the curvature of hypocotyl and flowerstalk towards the light source, whereas gravitropism can be observed by an upward curvature of hypocotyl and flowerstalk and downward curvature of the root. These tropism response patterns are outward evidence of a reaction to information concerning the status of the plant's environment (Poff *et al*., 1994).

This illustrates that, upon stimulation by conditions such as low temperatures, drought, decreased or increased light intensity, pathogen attack or mechanical stress, plant cell signal transduction pathways will up-regulate or down-regulate the expression of appropriate genes for the required physiological response such as changes in metabolism or direction of growth.

Plants not only depend on light to provide positional information to modulate developmental processes, but also as primary energy source. The regulation of photosynthetic components is therefore especially important to ensure the survival of the plant (Chory and Susek, 1994).

2.2. Signal Transduction and Gene Regulation in Plants

2.2.1. Light Regulation using the Phytochrome System and other Photoreceptors

For emerging seedlings whose development is governed by strategies to maximize photosynthetic capacity, light is one of the most important factors. Light is involved in chloroplast biogenesis, leaf meristem differentiation and the induction of many light regulated genes. Fig. 1 shows the very distinct phenotypes between etiolated seedlings grown in the dark and de-etiolated seedlings grown in light. Etiolated seedlings are characterized by elongated hypocotyls, closed cotyledons

and apical hooks, whereas light-grown seedlings exhibit short hypocotyls and open and expanded cotyledons.

This light-dependent development called photomorphogenesis is controlled by the combined action of several photoreceptor systems within the plant. They enable the plant to sense the quality, quantity, direction and duration of light and produce the appropriate physiological and developmental response. Photoreceptor systems are characterized by the wavelength of light that they perceive: red/far-red light (600-750 nm) is perceived by the phytochrome family, blue/UVA light (320-500 nm) by cryptochromes and phototropins and UVB (282-320 nm) by so far uncharacterized photoreceptor(s) (Sullivan and Deng, 2003).

Figure 1: Photographs of light- and dark-grown *A. thaliana* seedlings
The different phenotypes of light- and dark-grown *Arabidopsis* seedlings. Dark-grown seedlings are characterized by elongated hyocotyls, closed cotyledons and apical hooks, whereas seedlings grown in light show short hypocotyls and open, expanded and green cotyledons.

Phytochromes are the most intensively studied group of photoreceptors and are encoded by five genes in *Arabidopsis*, *PHYA-PHYE*. They are classified in two groups based on their stability (light labile and light stable). phyA is a type I phytochrome and is the most abundant phytochrome in dark grown seedlings which gets rapidly degraded by ubiquitination after exposure to light (Clough *et al*., 1999). phyB - phyE are type II, light stable phytochromes with phyB being most abundant in light-grown seedlings.

Phytochromes exist as soluble homodimers in the cell consistent of apoproteins covalently attached to a linear tetrapyrrole chromophore. *In vivo*, two photoreversible forms are present that show different conformations and absorption maxima. The red-light absorbing form (Pr) is

converted to the far-red light absorbing form (Pfr) upon exposure to red light. Pfr is considered the biologically active form that converts back to Pr upon absorption of far-red light or slowly in the absence of light (Wang and Deng, 2004).

Depending on the amount of light required, there are at least three physiological distinct modes of phytochrome action. In a very-low fluence response (VLFRs) plants respond to 0.1-1 μmol/m^2 of light, whereas a low fluence response (LFR) is typically between 1 to 1000 μmol/m^2 of light. A high fluence response (HIR) occurs generally at light influence of >1000 μmol/m^2. Of all these response modes, only LFRs show red/far-red reversibility and only LFRs and VLFRs show reciprocity. This describes the classic hallmarks of phytochrome response with exposure to red light inducing a certain effect which can be reversed by a subsequent far-red light treatment (R/FR reversibility) and the dependence on the total number of photons irrespective of the exposure duration (reciprocity) (Neff *et al*., 2000). Phytochrome apoprotein mutants have allowed an assessment of functions for the individual photoreceptors. Neff *et al*., 2000 consider the most important functions of phytochromes in a natural setting to be the end-of-day far-red and shade avoidance responses. Both are possible through the ability of the plant to sense the red/far-red ratio of light in their surroundings. By detecting the enrichment of far-red light at dusk, the end-of-day response affects flowering, whereas the enrichment of far-red light under a leaf canopy enables the plant to detect neighbors and induces shade avoidance. This response is characterized by the stimulation of elongation growth, often associated with reduced leaf development and increased apical dominance. Smith and Whitelam, 1997, have contributed the evolutionary success of flowering plants to the development of such mechanisms.

Specifically, the different phytochromes phyA - phyE play both distinct and overlapping roles within plant photomorphogenesis. phyA is involved in seed germination under various light conditions and responsible for seedling de-etiolation under continuous far-red light as well as promoting flowering under long day light conditions. phyB plays a role for seed germination and seedling de-etiolation under continuous red light and the shade avoidance response. phyC also promotes seed germination under continuous red light, while together with phyB both phyD and phyE are involved in the shade avoidance response. Additionally, phyE also plays a role in seed germination under low light conditions (Sullivan and Deng, 2003; Wang and Deng, 2004).

As underlying mechanisms for the observed functional differences between phytochromes, both conformational changes of various parts of the phytochrome proteins and interaction with distinct signaling pathways have been described (Wang and Deng, 2004). Additionally, the subcellular localization of phytochromes is affected by light. Both phyA and phyB are cytoplasmic in dark-grown plants and move into the nucleus upon exposure to light (Kircher *et al*., 1999 and

Yamaguchi *et al*., 1999). Yeh and Lagarias, 1998, could show that phyA from oat has serine/threonine kinase activity. Downstream phosphorylation substrates for phyA are still being investigated. So far, there have been no reports for kinase activities of phyB-phyE.

Cryptochromes and phototropins are the second major class of photoreceptors characterized by their ability to sense blue/UVA light. *Arabidopsis* has two genes encoding for cryptochromes (*CRY1* and *CRY2*) and the photoreceptor proteins contain a flavine adenine dinucleotide (FAD) and pterin (methenyltetrahydrofolate, MTHF) chromophore and a C-terminal extension with a so far unknown function. Also, two genes for phototropins (*PHOT1* and *PHOT2*) have been isolated from *Arabidopsis*. PHOT1 is a protein with two LOV domains (for light, oxygen and voltage-regulated proteins) at the amino terminus and a serine/threonine kinase domain at the carboxy terminus. Blue-light dependent phosphorylation activity has been described as the first genetic link between blue-light mediated phototropism and photophosphorylation (reviewed in Sullivan and Deng, 2003).

Examples of the involvement of different photoreceptors in specific developmental responses of *A. thaliana* are depicted in Fig. 2 (figure from Sullivan and Deng, 2003). Seed germination and shade avoidance are regulated by phytochromes phyA-phyE as described before. In a natural environment, etiolated followed by de-etiolated development allows the buried seedling to emerge through soil, reach light and switch to a developmental pattern optimal for photosynthesis (Fankhauser and Chory, 1997). This regulation of de-etiolation involves both phytochromes and cryptochromes (Sullivan and Deng, 2003). Phototropins are involved in the regulation of phototropism. The dependence on blue light of the directional curvature of plant organs towards light was first shown by Charles Darwin in 1881. Negative phototropism of roots (growth away from light) and positive phototropism of stems (growth towards light) has been described (Liscum, 2002). The curvature towards the light source results from increased growth of cells on the shaded side of stems and a corresponding decrease in growth of cells facing the light. Recently, a role also for phytochromes in phototropism in de-etiolated plants has been suggested (Liscum, 2002). Other processes regulated by phototropins include chloroplast movement and stomatal opening. Under low light conditions, chloroplasts accumulate on the upper surface of mesophyll cells, whereas in strong light they line up to minimize photo damage through excess light. Stomata are small pores in leafs and stems that regulate gas exchange by means of swelling or shrinking of the surrounding guard cells. Both phototropins in *Arabidopsis* have been described to regulate blue light-mediated chloroplast relocation and stomatal opening with some differences in their sensitivity to light (Sullivan and Deng, 2003). Further developmental processes influenced by photoreceptors depicted in Fig. 2 are the circadian clock and day length perception. The perception of day length

allows the plant to adjust development in anticipation of annual seasonal changes which for example influences the control of flowering (Yanovsky and Kay, 2002). This perception is mediated by an interaction of light-regulated pathways with circadian rhythms. The circadian clock is not completely isolated from environmental influences and must be reset (entrained) to be synchronized with the day/night cycle. In *Arabidopsis*, both phytochromes and cryptochromes contribute to the entrainment of the circadian clock, thereby also affecting flowering (Sullivan and Deng, 2003).

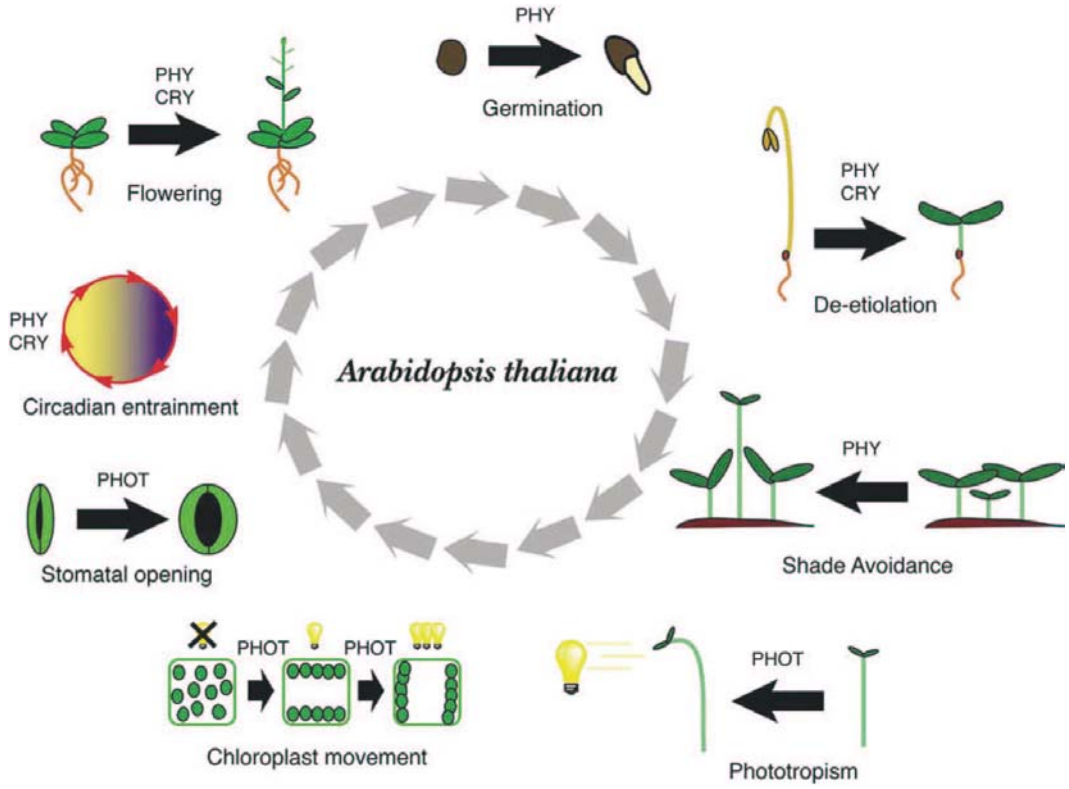


Figure 2: Light-regulated development in *A. thaliana*
Different aspects of development through the life cycle are influenced by classes of photoreceptor like phytochromes (PHY), cryptochromes (CRY) or phototropins (PHOT). Figure from Sullivan and Deng, 2003.

Upon perception of the light signal, signaling events downstream of the photoreceptors ultimately cause a change in gene expression. It appears that in *Arabidopsis* seedling development, phytochromes and cryptochromes ultimately regulate the same genes indicating a signal integration at some point during downstream signaling. Central to this integration are the COP/DET/FUS proteins which are essential for the repression of photomorphogenesis in the dark. One of these genes, *COP1* shows nuclear enrichment in the dark and has been shown to interact with transcription factors such as HY5 and HYH that act as positive regulators of

photomorphogenesis. COP1 acts as an E3 ubiquitin ligase and the interaction of COP1 with HY5 results in the targeting of HY5 for ubiquitin dependent degradation. A direct interaction of COP1 with CRY1 responsible for the blue light-mediated regulation of photomorphogenesis was also described. Six of the COP/DET/FUS proteins are part of an eight-subunit complex called the COP9 signalosome (CSN). The CSN is widely conserved in multicellular organisms and shows a homology to part of the 26S proteasome, a cellular protein-degradation device essential for the degradation of ubiquitinated proteins. CSN has been described as repressor complex and hypothesized roles for the CSN are in proteolysis and in chromatin remodeling as part of the regulation of gene expression during photomorphogenesis (Sullivan and Deng, 2003).

Additionally to the interaction with downstream signaling components, phytochromes can change their nucleocytoplasmic partitioning. This allows for direct interaction with transcription factors like PIF1 that negatively regulate chlorophyll biosynthesis (Huq *et al*., 2004), thereby raising the possibility of directly regulating gene expression rather than acting through a signaling cascade.

Of course, in many of the described responses, light is not the only factor controlling the final growth pattern observed in plants. Other factors such as circadian regulation and phytohormones are also involved in the interaction between many different environmental signals and intrinsic developmental programs in plants.

2.2.2. Regulation of Chlorophyll Biosynthesis in Plants

The clearest evidence for life on earth seen from outer space is the surge in chlorophyll abundance in terrestrial plants in springtime (Rüdiger, 1997). Thus, chlorophyll biosynthesis is one of the most important biochemical pathways known (Eckhardt *et al*., 2004). In addition to chlorophyll, three other major tetrapyrrole molecules are needed in higher plants. Chlorophyll, heme, siroheme and phytochromobilin are synthesized from a common precusor with the majority of the biosynthesis located in the chloroplasts.

Different amounts of each tetrapyrrole end product are needed in different cell types within the same plant and at different developmental stages. The requirement for heme is continuous in all cells to supply respiratory cytochromes and other essential hemoproteins such as peroxidases in different cellular compartments. In photosynthetic cells, chlorophyll is the major tetrapyrrole. It is confined to the chloroplasts and must be synthesized at a much higher level than heme. This demand is especially high upon the transfer of dark-grown seedlings into the light. However, many tetrapyrroles are readily excited by light and if unquenched can lead to the formation of free radicals which are toxic for the plant. Therefore, the need for a very strict regulation of

tetrapyrrole formation is obvious. The production of tetrapyrroles must exactly meet the demand while avoiding the accumulation of phototoxic intermediates (Cornah *et al*., 2003).

The biochemical pathway can be subdivided into three parts: the formation of the common precursor molecule 5-aminolevulinic acid (ALA), the formation of protoporphyrin IX from eight molecules of ALA and the formation of the main end products heme or cholorphyll involving the insertion of Fe^{2+} or Mg^{2+}, respectively. This division reflects both biochemical differences as well as differences in the control of the enzymatic steps. Although the production of each enzyme is individually and independently regulated, most genes required in chlorophyll biosynthesis show a light-induced and developmental-dependent expression profile. Additionally, particular enzymes mainly at the beginning of each section determine the metabolic flow and are targets for endogenous and environmental factors (Eckhardt *et al.*, 2004).

One of the last steps in chlorophyll biosynthesis requires the reduction of a porphyrin molecule into a chlorin. This reduction can occur by two entirely different mechanisms, one of which requires light and is catalyzed by a single enzyme and the other is independent of light, but requires at least three polypeptides. All chlorophyll-containing organisms appear to possess the light-dependent pathway and all except angiosperms and some algae are also able to utilize the light-independent route. Bacteriochlorophyll-containing photosynthetic bacteria contain only the light-independent pathway. As the sole reaction mechanism in angiosperms, the light-dependent reduction is catalyzed by an enzyme called NADPH-protochlorophyllide oxidoreductase (POR). POR is a plastid thylakoid membrane-associated protein that comprises a major portion of total etioplast membrane proteins. Together with bound NADPH and protochlorophyllide it builds a ternary complex. Exposure of the complex to light that is absorbed by the bound protochlorphyllide causes a rapid conversion of the pigment to chlorophyllide. Studies of POR function also revealed interesting results regarding the light-dependent regulation of the enzyme activity. Up to three separate genes named *POR-A*, *POR-B* and *POR-C* with different expression patterns encode for POR in angiosperms. In barley, POR-A is abundant in etiolated tissues, but both mRNA and protein appear to degrade rapidly upon exposure to light. *POR-B* is constitutively expressed in both etiolated and greening tissues and its mRNA is relatively stable. These results suggest that both gene products fulfill different roles dependent on the timing of their presence and activity in tissues. The major role for POR-A is to rapidly catalyze the formation of a sufficient amount of chlorophyll to allow nonphotochemical quenching to occur and to prevent photooxidative damage at the early stages of greening. After the initial formation of chlorophyll catalyzed by POR-A, the protein appears to be degraded by a specific, light-induced chloroplast protease. POR-B is then responsible for the continued chlorophyll formation until the full content

is reached and is involved in forming replacement chlorophyll (Beale, 1999). In *Arabidopsis*, three genes encoding for POR have been described with *POR-A* being dark-induced, *POR-B* constitutively expressed and *POR-C* light-induced (Eckhardt *et al*., 2004).

At the branch point between heme and chlorophyll biosynthesis, two chelatases compete for protoporphyrin IX. The insertion of Fe^{2+} is catalyzed by ferrochelatase which is a monomer encoded by a single gene. The reaction proceeds spontaneously and reversibly in the presence of protoporpohyrin IX and Fe^{2+} without the involvement of ATP or other energy source (Beale, 1999). Mg^{2+}chelation requires ATP and the enzyme appears to consist of three protein components (Beale, 1999). Two subunits were shown to form an activation complex under ATP hydrolysis and provide Mg^{2+}, while the third subunit carries the substrate (Eckhardt *et al*., 2004). The different properties of the two chelatases are important regulatory determinants of the relative flux through the two branches of the tetrapyrrole pathway. As the K_m for protoporphyrin IX of Mg-chelatase is lower than the value of Fe-chelatase, the kinetic properties suggest that Mg-chelatase has an advantage competing with Fe-chelatase for the same substrate. However, the activity of Mg-chelatase requires ATP. Therefore, the Mg branch of the pathway is favoured in the light, when ATP levels are higher, whereas the Fe-chelatase is more active in the dark (Cornah *et al*., 2003). This is also mirrored by the expression levels of the two chelatase genes with transcript levels of Mg-chelatase peaking in the light and the highest Fe-chelatase activity found a the beginning of darkness (Eckhardt *et al*., 2004). The allocation of substrate for both chelatases might also be regulated by a spatial separation of the enzymes. Ferrochelatase is located in both plastids and mitochondria with the plastids being the major heme-sythesizing organelle (Eckhardt *et al*., 2004). Within the chloroplast, ferrochelatase is thylakoid associated, whereas Mg chelatase can be found in the envelope, stroma and thylakoid fraction (Cornah *et al*., 2003). Another possibility for regulation is at the localization of gene expression. Ferrochelatases exist in two classes in higher plants with one produced ubiquitously throughout the plant and one only found in photosynthetic tissue. The first class of ferrochelatase is implicated in the synthesis of heme for respiratory cytochromes and hemoproteins involved in defense responses. Wounding or viral infection causes an up-regulation of theses genes which might provide the means to channel tetrapyrrole intermediates away form chlorophyll and into heme production. The expression in the photosynthetic tissue is dependent on functional plastids and is likely to be involved in heme production for photosynthetic cytochromes (Cornah *et al*., 2003).

Additionally to the regulation of the specific enzyme activities, coordination of tetrapyrrole and cognate apoprotein synthesis is important. Genes for the light-harvesting chlorophyll-*a/b*-binding

proteins (*Lhcb*) are up-regulated in light with signaling mechanisms that indicate the developmental status of the chloroplasts to the nucleus (Cornah *et al*., 2003).

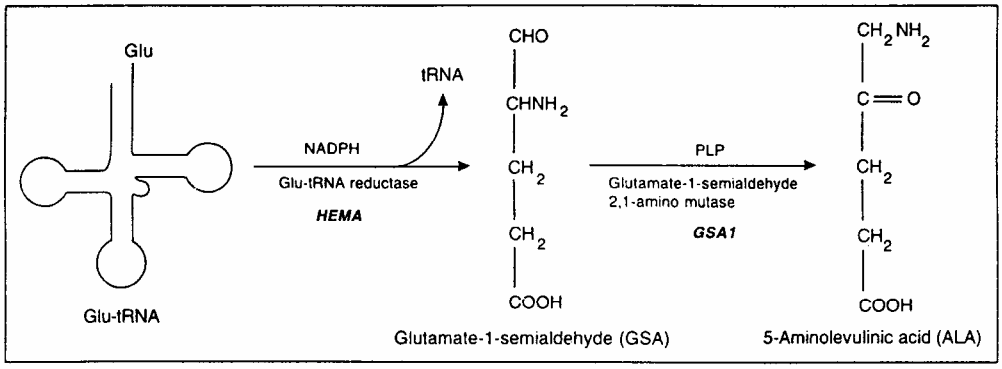


Figure 3: The C_5-pathway of ALA formation in *Arabidopsis* (Ilag *et al*., 1994)
Glu-tRNA reductase (GluTR) reduces Glu-tRNA^{Glu} to glutamate-1-semialdehyde (GSA). GSA is then converted to 5-aminolevulinic acid (ALA) by glutamate-1-semialdehyde-2,1-amino mutase (GSA-AM).

The formation of the common precursor 5-aminolevulinic acid (ALA) is the first committed step in the biosynthesis of tetrapyrroles and the rate-controlling point of the entire pathway (Beale and Castelfranco, 1974). In higher plants, ALA is formed via the C_5-pathway (Fig. 3) utilizing the five-carbon skeleton of glutamate (Jahn *et al*., 1992; Kumar *et al*. 1996b). In chloroplasts of plants, glutamate is acetylated to tRNA^{Glu} by glutamyl-tRNA synthetase (GluRS) and as Glu-tRNA^{Glu} represents the initial metabolite for the C5-pathway. Glu-tRNA reductase (GluTR) catalyzes the NADPH-dependent reduction to glutamate-1-semialdehyde (GSA) with the release of intact tRNA^{Glu}. Subsequently, GSA is converted into ALA by glutamate-1-semialdehyde-1,2-aminomutase (GSA-AM) using pyridoxal phosphate (PLP) as cofactor. As GluRS provides substrates for chloroplast protein synthesis as well, the reduction by glutamyl-tRNA reductase (GluTR) is considered the first step committed to tetrapyrrole synthesis. A role for GluTR as central control point for tetrapyrrole synthesis is supported by the observation that the expression of the *HEMA* genes is modulated by wide range of signals in different plants. The described regulatory signals range from hormones, circadian clock, plastid signals to light. In contrast, *GSA* genes encoding glutamate-1-semialdehyde-2,1-aminomutase respond only weakly if at all to the same signals (Cornah *et al*., 2003).

Both GluTR and GSA-AM are nuclear encoded and targeted to the chloroplast. In *Arabidopsis*, GSA-AM is encoded by a single gene, *GSA1,* which shows ubiquitous expression in five weeks old plants that increases upon exposure to white light (Ilag *et al*., 1994). At the beginning of this study, two genes encoding for GluTR had been described (Ilag *et al.*, 1994, Kumar *et al.*, 1996a).

HEMA1 showed expression in all tissues, but mostly in leafs and transcript levels were shown to increase following exposure to light (Ilag *et al*., 1994). The significance of the *HEMA1* gene for chlorophyll and heme biosynthesis was shown by transgenic plants expressing antisense *HEMA1* mRNA. All plants exhibited varying degrees of chlorophyll, heme and ALA deficiencies and a total lack of chlorophyll resulted in the failure to survive under normal growth conditions (Kumar and Söll, 2000). Additionally, the light-mediated induction of *HEMA1* gene expression was shown to be dependent on the presence of intact chloroplasts (Kumar *et al.,* 1999) indicating the requirement for plastid signaling. The level of *HEMA2* expression is lower than the level of *HEMA1*, expression is located predominantly in roots and flowers, has no light response (Kumar *et al.*, 1996a) and has since been shown to be inhibited by the presence of glucose or sucrose (Ujwal *et al*., 2002). Following the completion of the genomic sequence of *Arabidopsis,* analysis has shown that GluTR is encoded by three genes named *HEMA1* and *HEMA2* as well as *HEMA3*. No RT-PCR products for *HEMA3* have been detected and it has therefore been concluded that the gene is not a major contributor to ALA biosynthesis (McCormac and Terry, 2002). Recent DNA microarray based expression studies detected specific *HEMA3* mRNA in mature pollen and stamens (U. Vothknecht, personal communication).

Since the completion of the practical work for this study, several papers describing two independent mechanisms of down-regulation of chlorophyll biosynthesis involving GluTR were published. In darkness, chlorophyll synthesis in angiosperms is blocked at the light-dependent step of protochlorophyllide reduction. To avoid the over-accumulation of potentially phototoxic protochlorophyllide, a metabolic negative feedback mechanism through heme controls the activity of GluTR (Beale, 1999). The influence of heme on the rate of ALA synthesis was shown through mutants in tomato. These plants with a reduced heme breakdown show a reduced protochlorophyllide content due to reduction in ALA synthesis (Terry and Kendrick, 1999). Vothknecht *et al*., 1998, could show *in vitro* that the heme inhibition of GluTR activity requires the N-terminal 30 amino acids of the enzyme. Goslings *et al*., 2004, propose an independent, second mechanism through binding of a protein called FLU to GluTR. The flu mutant of *Arabidopsis* was isolated by Meskauskiene *et al*. 2001, and described as a heme oxygenase gene. The binding of FLU to GluTR was shown to be at different sites compared to the heme binding (Gosling *et al*., 2004). Both mechanisms provide means to inhibit the rate of ALA synthesis by interacting directly with GluTR.

2.2.3. Protein Phosphatase 2A and Signal Transduction

Reversible protein phosphorylation is an essential regulatory mechanism in many cellular processes. This post-translational modification can be used to alter properties like localization or activity of key regulatory proteins involved in specific pathways and recent studies have demonstrated the involvement of protein phosphorylation in almost all signaling pathways in plants. Phosphorylation and dephosphorylation of proteins is catalyzed by protein kinases and phosphatases, respectively. While early research had mostly focused on kinases, it is now apparent that protein phosphatases are highly regulated enzymes that play an equally important role in the control of protein phosphorylation (Janssens and Goris, 2001; Luan, 2003).

Protein kinases and phosphatases are divided into two major types by their substrate specificity: serine/theronine protein kinases and phosphatases as well as protein tyrosine kinases and phosphatases. Protein phosphatases are further defined in at least three distinct subfamilies based on biochemical and structural characteristics. The family of PTPs contains both tyrosine-specific and dual-specificity phosphatases. Ser/Thr phosphatases themselves are divided into PPP and PPM subgroups (Luan, 2003). Historically, the distribution of Ser/Thr phosphatases into subgroups was based on pharmacological properties. Type 1 phosphatase PP1 prefers the beta-subunit of phosphorylase kinase as substrate and is inhibited by two peptide inhibitors, inhibitor 1 and 2. The group of type 2 phosphatases (PP2s) is insensitive to inhibitor 1 and 2 and preferentially dephosphorylates the alpha-subunit of phosphorylase kinase. The PP2 enzymes are further distinguished by their dependence of divalent cations. PP2C is regulated by Mg^{2+}, PP2B by Ca^{2+}, whereas PP2A, like PP1, does not require cations for enzyme activity (Cohen, 1989). More recent sequence and structural analysis of these enzymes have demonstrated a closer relationship of PP1, PP2A and PP2B and therefore, they have been defined as PPP family. Additionally, the PPP family also includes a number of non-classical isoforms, like PPX/PP4, that are found in a large variety of organisms and show similarities to PP1 and PP2A. PP2C and several other Mg^{2+}-dependent Ser/Thr phosphatases have been grouped into the PPM family (Cohen, 1997; Luan, 2003).

Protein phosphatase 2A (PP2A) is a ubiquitous enzyme that shows a high degree of conservation across different species. The core enzyme is a dimer consisting of a 36 kDa catalytic subunit and a regulatory A-subunit. A third regulatory B-subunit can be associated with this core structure (Fig. 4). The regulatory B-subunits are more heterogenous and grouped into B, B', B" and, in some organisms, B"' subfamilies (Janssens and Goris, 2001). The regulatory A-subunit functions as scaffold within the holoenzyme and allows a regulatory B-subunit to bind.

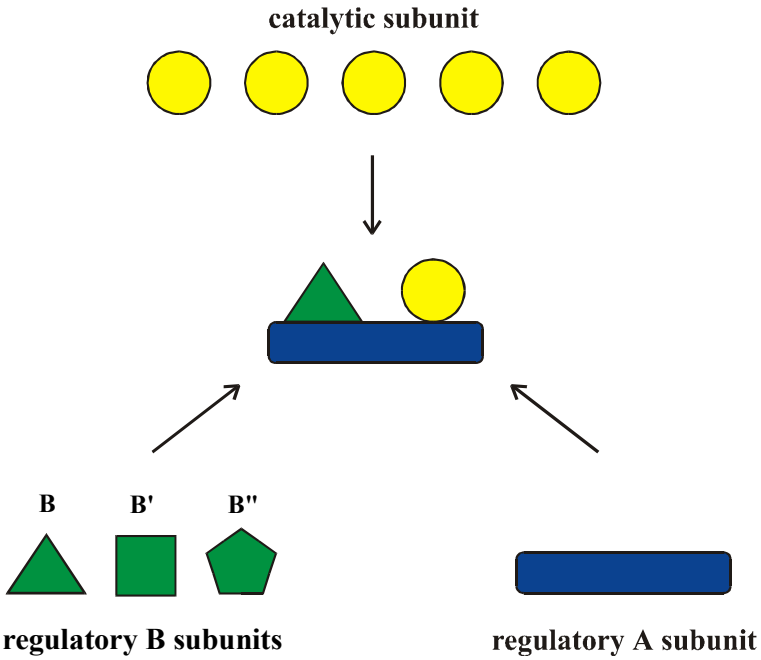


Figure 4: Protein Phosphatase 2A in *A. thaliana*
The core dimer consists of one of five catalytic C-subunits and a regulatory A-subunit, whereas addition of another regulatory B-subunit builds the holoenzyme. Genes for three A-subunits, two B-subunits, eight B'-subunits and nine B"-subunits are annotated within the genome of *Arabidopsis*.

B-subunits are differentially expressed and can be responsible for controlling the localization, activity and specificity of the different holoenzymes (Luan, 2003). This could result in a number of different isoforms of the PP2A holoenzyme that are expressed in a cell-, tissue- or developmental stage-specific manner. Also, the enzymatic activity of the catalytic subunit can be modulated positively or negatively depending on the regulatory subunits within the complex. The binding of the A-subunit alone can influence the activity of the catalytic subunit even in the absence of a B-subunit (Turowski *et al*., 1997). The analysis of the *Arabidopsis* genome has revealed genes for three A-subunits, a total of 17 B-subunits (two B, eight B' and nine B") and five catalytic C-subunits. Therefore, a total of 255 possible heterotrimeric PP2A isoforms can be predicted from the *Arabidopsis* genome (Zhou *et al*., 2004).

In mammalian systems, two catalytic subunits named isoforms α and β which share a high degree of identity have been described (Janssens and Goris, 2003). Ronne *et al*., 1991, described three isoforms of C-subunits in yeast; the very closely related PPH21 and PPH22 as well as PPH3. In contrast, the complexity of the PP2A catalytic subunit family is much higher in plants than in other eukaryotes. Five isoforms of catalytic subunits have been described in *A. thaliana* that can be grouped in two subfamilies on the basis of their amino acid sequence identity (Ariño *et al*., 1993; Casamayor *et al*., 1994; Pérez-Callejón *et al.,* 1998; Stamey and Rundle, 1996). RNA blots have shown that the genes for all five subunits are ubiquitously expressed, however the levels of

expression vary (Ariño *et al*., 1993; Pérez-Callejón *et al.,* 1993). The significance of this multiplicity in plants that also includes an increased number of PP1 isoforms in *Arabidopsis* is presently unknown.

In animals and yeast, PP2A functions in numerous signaling pathways including regulation of DNA replication, cell cycle progression, termination of translation, early embryogenesis and apoptosis (Janssens and Goris, 2001). However, relatively little was know about the role of PP2A in plant signaling pathways at the beginning of this study. Studies with pharmacological approaches using PP1/PP2A specific inhibitors like okadaic acid or cantharidin have implicated a PP1/PP2A requirement for the activation of metabolic enzymes like nitrate reductase and sucrose phosphate synthase, which are important in carbon and nitrogen metabolism. Inhibitor analysis also suggested that PP1/PP2A is involved in guard cell ion channel regulation, gene expression and developmental processes (Luan, 2003). Furthermore, PP2A has been implicated in cold responses, hormone and pathogen responses and root cortical cell elongation (Zhou *et al*., 2004). Virus-induced silencing of the first subfamily of PP2A catalytic subunits in tobacco, determined a role for PP2A enzymes as negative regulators of plant defense responses (He *et al*., 2004). Regulatory subunit mutants have been used to further elucidate the function of PP2A. *ton2* carries a mutation within a gene encoding a B"-subunit and displays abnormalities in cell shapes that result in changes in the overall plant morphology. The mutant shows cortical microtubule disorganization and increased auxin content and ethylene production (Zhou *et al*., 2004). The *rcn1* mutant was originally isolated for its altered response to an auxin transport inhibitor. It was characterized as a mutation within a regulatory A-subunit (Garbers *et al*., 1996). The phenotypic expression of the mutation includes defects in differential cell elongation responses including gravitropism, in polar auxin transport and in abscisic acid response. Recently, the availability of sequenced-indexed mutant collections in *Arabidopsis* (Alonso *et al*., 2003) was used to examine functions of the two other regulatory A-subunits. However, the mutants did not resemble *rcn1*. On plants carrying a single mutation in one of the A-subunits no obvious phenotypic alteration under normal growth conditions was observed. These results imply that the A-subunits fulfill separate but partially overlapping biological roles with the RCN1 protein playing a cardinal role in overall PP2A regulation (Zhou *et al*., 2004). Physiological and biochemical assays show that the wild-type *RCN1* encoded A-subunit functions as a positive regulator of the PP2A holoenzyme (Deruère *et al*., 1999).

Considering the differences in number of catalytic subunits and in described functions between animals and plants, further work will be required to elucidate the biological significance of PP2A function in plants.

2.3. *Arabidopsis thaliana*: A Model System for Plant Research

A. thaliana (see Fig. 5) serves as a model system for plant research for several reasons. The plant itself is of relatively small size and the generation time of five to six weeks under optimal growth conditions is rapid in comparison to other plants. Also, *Arabidopsis* has the ability to grow well in controlled conditions either on soil or defined media, shows prolific seed production and the ease of cross- and self-fertilization is advantageous.

Figure 5: Photographs of *Arabidopsis* plants
Life stages of the *Arabidopsis* plant from the first emerging leaves through the rosette state up to the flowering stage. Under optimal growth conditions, the generation time from the germination of seedlings until the harvest of seeds is only five to six weeks.

Its diploid genome is relatively small (125 Mb) compared to other plants and consist of 5 chromosomes. Since the completion of the *Arabidopsis* genome project (The Arabidopsis Genome Initiative, 2000), sequence information is easily available and there are well established transformation methods that allow the study of gene functions in transgenic plants. Additionally,

there are several collections of mutant plants available through the Arabidopsis Biological Resource Center (ABRC). Among others, the Salk Institute Genomic Analysis Laboratory (SIGnAL) created a sequence-indexed library of mutations in the *Arabidopsis* genome using collections of *Agrobacterium* T-DNA transformed plants. These collections contain over 225,000 independent insertion events, thereby saturating the entire genome of *A. thaliana* (Alonso *et al*., 2003). A multinational research community of academic, government and industry laboratories shares information over several websites like the TAIR - The Arabidopsis Information Resource at http://www.arabidopsis.org.

For all these reasons, *A. thaliana* has been a model system for plant research for many years and was chosen as organism to perform the analyses described within this study.

2.4. Aim of this Study

The initial aim of this thesis was the elucidation of the molecular basis of light-dependent chlorophyll biosynthesis at the gene level. Light regulation-mediating promoter elements of the *HEMA1* gene encoding the first enzyme of chlorophyll biosynthesis, Glutamyl-tRNA reductase, were sought to be identified. For this purpose, *HEMA1* promoter deletions fused to the reporter gene GUS were tested for their light-dependent transient expression in protoplasts as well as for stable expression in transgenic plants.

The second goal was to determine the role of protein phosphatase 2A (PP2A) in *HEMA1* gene expression and subsequently in the general control of cellular processes in *Arabidopsis*. Two different experimental designs were employed. A yeast two-hybrid screen using two catalytic subunits of *A. thaliana* PP2A as bait proteins was chosen to find possible interaction partners and thereby identifying targets for PP2A in *Arabidopsis*. The second approach used a PCR-based screening for T-DNA mutants within PP2A catalytic subunit genes in *Arabidopsis*. Phenotypes of isolated mutants could allow conclusions about the involvement of PP2A in cellular processes and thereby further defining multiple functions for protein phosphatase 2A in this plant.

3. Materials and Methods

3.1. Materials

3.1.1. Bacterial and Yeast Strains, Plant Material

Strain		Reference
Agrobacterium tumefaciens:		
GV3101 (pMP90)	C58C1, Rif^R, Gm^R	Koncz and Schell, 1986
Escherichia coli:		
DH5α	F^-, ϕ80d*lacZ* ΔM15, Δ(*lacZYA-argF*), U169, *endA1*, *recA1*, *hsdR17*, (r_k^--m_k^+), *deoR*, *thi*-1, *supE44*, λ-, *gyrA96*, *relA1*	Sambrook *et al.*, 1989
JS5	*araD139*, Δ(*ara, leu*)7697, $\Delta(lac)_{\chi 74}$, *galU*, *galK*, *hsdR2* (rk^- mk^-), *mcrA*, *mcrBC*, *rps*L (*Strr*) *thi*, *recA1/F'*::Tn10 (Tet^r) *proAB*, *lac*I^q, *lacZM15*	Bio-Rad Laboratories, Hercules, CA
Saccharomyces cerevisiae:		
Y190	*MATa, leu2-3,112, ura3-52, trp1-901, his3-Δ200, ade2-101, gal4Δgal80Δ URA3 GAL-lacZ, LYS GAL-HIS3, cyh*^{r}	Harper *et al.*, 1993

Plants:
Arabidopsis thaliana (L.) Heynh., ecotype Columbia

3.1.2. Chemicals

1 kb DNA Ladder	Gibco Life Technologies, Gaithersburg, MD
[α-^{32}P] dCTP (10 mCi/ml)	Amersham, Arlington Heights, IL
[α-^{35}S] dATP (10 mCi/ml)	Amersham, Arlington Heights, IL
Acetone	J.T. Baker, Phillipsburg, NJ
Acrylamide	American Bioanalytical, Natick, MA
Agar	USB, Cleveland, OH and Difco, Sparks, MD
Agarose	USB, Cleveland, OH
Amino Acids (see yeast media)	Sigma, St. Louis, MO
3-Amino-Triazole (3-AT)	Sigma, St. Louis, MO
Ammonium Persulfate (APS)	American Bioanalytical, Natick, MA
Ampicillin	USB, Cleveland, OH
Bacto-Agar	Difco, Sparks, MD
Benzylamino Purine	Sigma, St. Louis, MO
Bisacrylamide (N,N'-Methylene Bisacrylamide)	Serva, Heidelberg, Germany
Boric Acid	J.T. Baker, Phillipsburg, NJ
Bovine Serum Albumin (BSA)	Roche Boehringer Mannheim, Indianapolis, IN
5-Bromo-4-Chloro-3-Indolyl-β-D-Galactopyranoside (X-Gal)	Roche Boehringer Mannheim, Indianapolis, IN
5-Bromo-4-Chloro-3-Indolyl-β-D-Glucuronide (X-Gluc)	Clontech, Palo Alto, CA
5-Bromo-4-Chloro-3-Indolylphosphate (X- phosphate, BCIP)	Sigma, St. Louis, MO
Bromphenol Blue	USB, Cleveland, OH
Bradford Reagent	Biorad, Hercules, CA

Cantharidin	Sigma, St. Louis, MO
Chloroform	J.T. Baker, Phillipsburg, NJ
Coomassie Brilliant Blue (G-250)	Sigma, St. Louis, MO
3-Cyclohexylamino-1-Propane Sulfonic Acid (CAPS)	Serva, Heidelberg, Germany
Deoxyribonucleoside Triphosphates (dATP, dCTP, dGTP, dTTP)	Roche Boehringer Mannheim, Indianapolis, IN
Dideoxyribonucleoside Triphosphates (ddATP, ddCTP, ddGTP, ddTTP)	Roche Boehringer Mannheim, Indianapolis, IN
Diguanosine Triphosphate (GMP) (P^1-5'-(7-methyl)-guanosine-P^3-5'-guanosine-triphosphate)	Roche Boehringer Mannheim, Indianapolis, IN
Dimethylformamide (DMF)	Aldrich, Milwaukee, WI
Dimethyl Sulfoxide (DMSO)	Mallinckrodt, Paris, KY
Dithiothreitol (DTT)	American Bioanalytical, Natick, MA
Ethylenediaminetetraacetic Acid (EDTA)	J.T. Baker, Phillipsburg, NJ
Ethanol	J.T. Baker, Phillipsburg, NJ
Ethidium Bromide	USB, Cleveland, OH
Ficoll	Sigma, St. Louis, MO
Formamide	American Bioanalytical, Natick, MA
Gentamycin	Sigma, St. Louis, MO
Glycine	American Bioanalytical, Natick, MA
Heparin	Sigma, St. Louis, MO
Hygromycin	Sigma, St. Louis, MO
Isopropyl-β-D-Thiogalactopyranoside (IPTG)	American Bioanalytical, Natick, MA
Kanamycin	Sigma, St. Louis, MO
Lambda DNA	New England Biolabs, Beverly, MA
N-Lauroylsarcosine (Sarkosyl)	Sigma, St. Louis, MO
Levamisole	Sigma, St. Louis, MO
β-Mercaptoethanol	American Bioanalytical, Natick, MA
Methanol	J.T. Baker, Phillipsburg, NJ
4-Methyl-Umbelliferone (MU)	Clontech, Palo Alto, CA
4-Methyl-Umbelliferyl-β-D-Glucuronide (MUG)	Sigma, St. Louis, MO
Milk Powder (dry)	Nestle Carnation, Glendale, CA
Murashige and Skoog Salts (MS)	Sigma, St. Louis, MO
Nitro-Blue Tetrazolium (NBT)	Sigma, St. Louis, MO
Phenol	American Bioanalytical, Natick, MA
Phenyl Methyl Sulfonyl Fluoride (PMSF)	Sigma, St. Louis, MO
Peptone	Difco, Sparks, MD
Polyethylene glycol 3350 or 6000 (PEG)	Sigma. St. Louis, MO
Polyvinylpyrrolidone (PVP)	Sigma, St. Louis, MO
Ponceau S	Sigma, St. Louis, MO
Potassium Chloride	J.T. Baker, Phillipsburg, NJ
RNasin	Promega, Madison, WI
Salmon Sperm DNA	Sigma, St. Louis, MO
SDS-PAGE Molecular Weight Standards	Biorad, Hercules, CA
Sodium Chloride	J.T. Baker, Phillipsburg, NJ
Sodium Dodecyl Sulfate (SDS)	American Bioanalytical, Natick, MA
Sodium Hydroxide	J.T. Baker, Phillipsburg, NJ
Spectinomycin	Sigma, St. Louis, MO
Streptomycin	Sigma, St. Louis, MO
Succinic Acid	ICN Biomedicals Inc., Aurora, OH
Sucrose	Sigma, St. Louis, MO

N, N, N', N'-Tetramethyl Ethylenediamine (TEMED)	American Bioanalytical, Natick, MA
Trishydroxymethyl Aminomethane (Tris, Trizma Base)	American Bioanalytical, Natick, MA
Triton X-100	Sigma, St. Louis, MO
Tryptone	Difco, Sparks, MD
Tween-20	USB, Cleveland, OH
Urea	American Bioanalytical, Natick, MA
Xylene Cyanole FF	Sigma, St. Louis, MO
Yeast Extract	Difco, Sparks, MD
Yeast Nitrogen Base	Difco, Sparks, MD

3.1.3. Enzymes, Antibodies and Kits

Cellulase	Sigma, St. Louis, MO
Calf Intestinal Alkaline Phosphatase (CIP)	New England Biolabs, Beverly, MA
DNAse I	Roche Boehringer Mannheim, Indianapolis, IN
Driselase	Sigma, St. Louis, MO
Klenow Fragment of DNA Polymerase	Roche Boehringer Mannheim, Indianapolis, IN
Lysozyme	Sigma, St. Louis, MO
Mazerozyme R-10	Serva, Heidelberg, Germany
Pectolyase	ICN Biomedicals, Aurora, OH
Restriction Enzymes	New England Biolabs, Beverly, MA
Reverse Transcriptase (AMV)	Roche Boehringer Mannheim, Indianapolis, IN
Ribonuclease A (RNAse A)	Roche Boehringer Mannheim, Indianapolis, IN
T3 RNA Polymerase	Promega, Madison, WI
T4 DNA Ligase	New England Biolabs, Beverly, MA
T7 RNA Polymerase	gift from Dr. M. Ibba
Taq DNA Polymerase	Promega, Madison, WI and Roche Boehringer Mannheim, Indianapolis, IN
Anti-DIG Fab Fragment Alkaline Phosphatase Conjugate	Roche Boehringer Mannheim, Indianapolis, IN
Anti-PP2A C Antibody (antibody against carboxy peptide of catalytic subunit of *Xenopus* produced in rabbit)	gift from J. Deruère (Deruère et al., 1999) (prepared by Todd Stukenberg and Marc Kirschner)
Anti-HA Antibody (Mouse)	Biovision, Mountain View, CA
Anti-Mouse IgG_{2b} Peroxidase Conjugate	USB, Cleveland, OH
Anti-Rabbit Antibody with Peroxidase Conjugate	USB, Cleveland, OH
DIG RNA Labeling Mix	Roche Boehringer Mannheim, Indianapolis, IN
LumiGLO Chemiluminescent Substrate	KPL, Gaithersburg, MD
QIAquick Gel Extraction Kit	Qiagen, Valencia, CA
QIAquick Nucleotide Removal Kit	Qiagen, Valencia, CA
QIAquick PCR Purification Kit	Qiagen, Valencia, CA
*Redi*prime DNA Labeling System	Amersham, Arlington Heights, IL
Sequenase Kit	USB, Cleveland, OH

3.1.4. Filters and Membranes

Hybond N^+ Membrane	Amersham, Arlington Heights, IL
Miracloth	Calbiochem, San Diego, CA
Nitrocellulose Filters	Schleicher & Schuell, Keene, NH
Protran Nitrocellulose Membrane	Schleicher & Schuell, Keene, NH
Sterile Filters (0.2 μm, 0.45 μm)	Pall Gelman Sciences, Ann Arbor, MI
Whatman 3MM Paper	Whatman, Clifton, NJ
Whatman #1 Filter Paper	Whatman, Clifton, NJ

3.1.5. Media

3.1.5.1. Antibiotics, Hormones and Stock Solutions

	Stock Solution	Final Solution
3-Amino-triazole (3-AT)	84 mg/ml (1M)	25 μmol/ml
Ampicillin	100 mg/ml	100 μg/ml to 150 μg/ml
5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal)	20 mg/ml in dimethylformamide (DMF)	80 μg/ml
5-Bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc)	10.4 mg/ml (20 mM) in dimethylformamide (DMF)	see 3.2.11.1.
5-Bromo-4-chloro-3-indolyl-phosphate (X- phosphate, BCIP)	50 mg/ml in dimethylformamide	see 3.2.12.3.
Cantharidin	50 mM	5 μM
Gentamycin	50 mg/ml	50 μg/ml
Hygromycin	15 mg/ml	15 μg/ml
Kanamycin	50 mg/ml	50 μg/ml
4-Methyl-umbelliferone (4-MU)	19.8 mg/ml (0.1 M) in H_2O	see 3.2.11.2.
4-Methyl-umbelliferryl glucoronide (4-MUG)	35.2 mg/ml (0.1 M) in dimethylformamide	see 3.2.11.2.
Nitro-blue tetrazolium (NBT)	50 mg/ml in 70% v/v H_20/dimethylformamide	see 3.2.12.3.
Spectinomycin	100 mg/ml	100 μg/ml
Streptomycin	50 mg/ml	50 μg/ml

3.1.5.2. Bacterial Media

LB:	10	g/l	Bacto Tryptone
	5	g/l	Yeast Extract
	10	g/l	NaCl
	1.5%	(v/w)	Bactoagar

Dissolved in distilled water, adjusted pH to 7.5 and autoclaved for 20 min.

3.1.5.3. Plant Media

Cantharidin plates:	½	Murashige & Skoog Salts (2.22 g/l media)
	1% (w/v)	Sucrose
	1.5% (w/v)	Agar

Dissolved in distilled water, adjusted pH to 5.7-5.9 with KOH and autoclaved for 20 min. Directly after autoclaving, 100 µl of 50mM cantharidin stock solution was added and stirred into solution to achieve a final concentration of 5 µM.

MS:	½	Murashige & Skoog Salts (2.22 g/l media)
	1% (w/v)	Sucrose
	1% (w/v)	Agar

Dissolved in distilled water, adjusted pH to 5.7-5.9 with KOH and autoclaved for 20 min.

Rooting plates:	½	Murashige & Skoog Salts (2.22 g/l media)
	1% (w/v)	Sucrose
	0.8% (w/v)	Agar

Dissolved in distilled water, adjusted pH to 5.7-5.9 with KOH and autoclaved for 20 min.

Selection plates:	½	Murashige & Skoog Salts (2.22 g/l media)
	3% (w/v)	Sucrose
	0.8% (w/v)	Agar

Dissolved in distilled water, adjusted pH to 5.7-5.9 with KOH and autoclaved for 20 min. After autoclaving, the media was allowed to cool down and 15 µg hygromycin per ml media was added.

3.1.5.4. Yeast Media

Amino Acid Mix:

Adenine	final concentration in media:	40	mg/l
L-Arginine	final concentration in media:	20	mg/l
L-Glutamic Acid	final concentration in media:	100	mg/l
Isoleucine	final concentration in media:	30	mg/l
L-Lysine	final concentration in media:	30	mg/l
L-Methionine	final concentration in media:	20	mg/l
L-Phenylalanine	final concentration in media:	50	mg/l
L-Serine	final concentration in media:	400	mg/l
L-Tyrosine	final concentration in media:	30	mg/l
L-Valine	final concentration in media:	150	mg/l

Amino Acid Solutions:
Each dissolved in dH_2O and filter sterilized using 0.2 μm filter.

L-Aspartic Acid	final concentration in media:	100	mg/l
L-Histidine	final concentration in media:	20	mg/l
L-Leucine	final concentration in media:	60	mg/l
L-Threonine	final concentration in media:	200	mg/l
L-Tryptophan	final concentration in media:	40	mg/l
Uracil	final concentration in media:	20	mg/l

AT-plates:
3-AT solution at a final concentration of 25 μmol/ml was added to the apropriate selection plates.

10 x BU salts:

70 g/l	Na_2HPO_4 x 7 H_2O
30 g/l	NaH_2PO_4

Added to distilled water to achieve final volume, adjusted pH to 7.0 and autoclaved.

Minimal plates:

	Amino Acid Mix
	(to achieve final concentrations as mentioned above)
2% (w/v)	Difco Agar

Dissolved in distilled water to achieve final volume and autoclaved. Volume of water used depended on amino acid solutions necessary for selection that were added to media after autoclaving.
After autoclaving, the following was added to media:

6 ml	Yeast Nitrogen Base
8 ml	50% Glucose Solution

Additionally, appropriate amino acid solutions were added as needed for selection purposes in same concentrations as mentioned before.

SC:

10	g/l	Succinic Acid
6	g/l	NaOH
		Amino Acid Mix
		(to achieve final concentrations as mentioned above)
2% (w/v)		Agar (not added for liquid media)

Dissolved in distilled water and autoclaved for 20 min. After autoclaving the following was added:

40	ml/l	50% Glucose Solution
30	ml/l	Yeast Nitrogen Base
100	mg/l	L-Aspartic Acid
20	mg/l	L-Histidine
60	mg/l	L-Leucine
200	mg/l	L-Threonine
40	mg/l	L-Tryptophan
20	mg/l	Uracil solution

If plates were used for selection purposes, the appropriate amino acid solution or solutions were omitted from the media and the final volume was adjusted. The plates were labeled to indicate the sugar source and the missing amino acid or amino acids (for example Glu-trp).

X-gal plates:
X-gal solution and 10 x BU salts were added to the appropriate selection plates with the final concentrations being 80 μg/ml and and 1 x, respectively.

Yeast Nitrogen Base:

223.3 g/l	in distilled water and filter sterilized (0.2 μm).

YPD: 1% (w/v) Yeast Extract
2% (w/v) Peptone
2% (w/v) Agar (not added for liquid media)
Dissolved in 960 ml of distilled water and autoclaved for 20 min. After autoclaving 40 ml of a 50% glucose solution was added.

3.1.6. Plasmids and Oligonucleotides

Plasmids

pCG: Vector carries *Hind*III fragment from pRTL2-GUS (Restrepo *et al*., 1990) in a pBluescript (Stratagene) background (A.M. Kumar and D. Söll, unpublished data), containing the coding sequence for the reportergene β-glucuronidase (GUS) under control of the CaMV 35S promoter

pCIT20: Binary vector with multiple cloning site, Hy^R, Sp^R, Str^R, replication functions for both *E. coli* and *A. tumefaciens*, contains right T-DNA border for initiation of T-DNA transfer into plant cells (Ma *et al.*, 1992)

TA vector: Vector based on pBluescript II KS +/- (Stratagene) with additional MCS (multiple cloning site) containing ribosome binding site (SD-site) for IPTG induced overexpression, two *Bam*H I sites for release of insert and two *Xcm*I sites that create T-overhang and allow for direct cloning of PCR products (Kwak and Kim, 1995)

pAH6: cDNA of *Arabidopsis HEMA1* in pBluescript II KS +/- vector (Stratagene), inserted into *Eco*RI site (gift from Dr. A. Madan Kumar)

Oligos

C1-f: 5'- GA GAC GAC GGA TCC AGA TGC CGT TAA ACG GAG ATC -3'
C1-r: 5'- G AAA AAA CTC GAG ATC ACA AAA AAT AAT CAG GGG -3'
C3-f1: 5'- GAC GAA TGG ATC CAA ATG GGC GCG AAT TCC ATT CCG -3'
C3-r1: 5'- G AGC AAC CTC GAG ACA ATG CAT AGG ACC AGA ATC -3'
C3-f2: 5'- CCG GAT CGG ACG AGA GAC GAA TGG ATC CAA ATG GGC -3'
C3-r2: 5'- GTT GAA TTT TAA ACA GAG CAA CCT CGA GAC AAT GCA -3'

pACT-f: 5'- A ACT ATC TAT TCG ATG ATG -3
pACT-r: 5'- GC ACA GTT GAA GTG AAC -3

EN205: 5'- AGA AGC ACG ACG GCT GTA GAA TAG GA -3'
EN8130: 5'- GAG CGT CGG TCC CCA CAC TTC TAT AC -3'
PC1-1: 5'- GAG CTA TT CGT ATT GGT GGT AAT GC -3'
PC1-2: 5'- ATG ATG GGT TTT TCG TCT TTG GTA TC -3'
PC2-1: 5'- ATA TAG GAT CGG TGG TGG CTT TTC AA -3'
PC2-2: 5'- TTT TTA TAG CAA TCA CCT CAA CAA CA -3'
PC2-3: 5'- AGC AGC TGA TGG AGT GTA AAC CGT TAT -3'
PC2-4: 5'- AAA AAT AAT CAG GGG TCT TCC GAG TAG T -3'
PC3-1: 5'- TCC ATC CAA TTT AGG GTT AGG ACT TC -3'
PC3-2: 5'- TTC TCA CAT GAA ACC ACA TTC TGA TG -3'
PC3-3: 5'- GAG CAT TAT GCG AGA AAG CCA AGG AGA T -3'
PC3-4: 5'- GAT GCA GAA GGG AGA AGA AGA AGA AGC T -3'
PC4-1: 5'- GAA GGA AAG AAG ACG ACG ACG CAC TA -3'
PC4-2: 5'- TGA TTT TAA AGA GAA GCA ACC TGG AA -3'
PC5-1: 5'- AAA TCC ATG GAG TGA TGA TGA TGA TA -3'
PC5-2: 5'- ACC AGA ATC TTT GCA CAC AAA AGT TT -3'

PC5-3:	5'- GAA ACG GAG GTG AAG ATG TTG TGT GAG C -3'
PC5-4:	5'- CAG AAT CTT TGC ACA CAA AAG TTT ACG G -3'

oligo dT (5 A_{260} U)	Roche Boehringer Mannheim, Indianapolis, IN

3.1.7. Yeast Two-Hybrid System

For this study, the yeast two-hybrid system used was obtained from the *Arabidopsis* Biological Resource Center based at Ohio State University with support from the NSF/DOE/USDA Collaborative Research in Plant Biology Program, Research Collaboration Group in Plant Protein Phosphorylation (USDA 92-37105-7675). The yeast strain Y190 and the bait and library vectors pAS1 and pACT are described in Harper *et al.*, 1993 and in Durfee *et al.*, 1993, respectively.
Two libraries were used in this study. CD4-10 was developed by Dr. S. Elledge using random-primed mRNA isolated from mature *Arabidopsis* leaves and roots. CD4-22 was prepared by Dr. J. Kim and Dr. A. Theologis and was made using mRNA isolated from 3 days-old etiolated seedlings.

3.1.8. Other Materials

Kodak BioMax MS/MR Film	Amersham, Arlington Heights, IL
Kodak X-Omat Film	Amersham, Arlington Heights, IL

3.2. Methods

3.2.1. DNA Preparations

3.2.1.1. Isolation of Plasmid DNA from *Escherichia coli*

Boiling Method:

TE:	10 mM	Tris, pH 8
	1 mM	EDTA

Triton-X-Lytic Buffer:

8% (w/v)	sucrose
0.5% (v/v)	Triton-X100
50 mM	EDTA, pH 8.0
10 mM	Tris, pH 8.0

Pinch of lysozyme added.

Bacterial o/n cultures were filled into 1.5 ml Eppendorf tubes and spun in benchtop centrifuge at full speed for 40 sec. The supernatant was discarded, the bacterial pellet was resuspended in 370 µl of Triton-X Lytic Buffer and the pellet was dissolved. Tubes were kept on ice for 5 - 10 min. The samples were heated at 95° C for 1 min and then centrifuged at full speed for 10 - 20 min. The resulting pellet was removed using a toothpick, 80 µl of 10 M NH_4OAc and 500 µl of isopropanol were added to the tube and the samples were mixed. To precipitate the DNA, the samples were put on ice for 10 min and then spun at full speed for 10 min. The supernatant was aspirated and the pellet air dried. The pellet was resuspended in 200 µl of TE.

Alkaline Lysis Protocol:

GTE:	50 mM	glucose
	25 mM	Tris-HCl, pH 8
	10 mM	EDTA

NaOH/SDS solution (made fresh):

200 mM	NaOH
1% (w/v)	SDS

3 M KAc (pH ~ 5.5):	294 g/l	potassium acetate
	50 ml/l	90% formic acid (1.18 M final concentration)

Solved in distilled H_2O.

TE:	10 mM	Tris, pH 8
	1 mM	EDTA

Bacterial o/n cultures were filled into 1.5 ml Eppendorf tubes and spun in a benchtop centrifuge at full speed for 20 sec. The supernatant was discarded, the bacterial pellet was resuspended in 100 µl of GTE and the tubes were left at room temperature for 5 min. 200 µl of NaOH/SDS solution were added and the samples were put on ice for 5 min. 150 µl of KAc solution were added, mixed for 2 sec and the tubes were left on ice for an additional 5 min. Samples were centrifuged for 3 min at full speed. 400 µl of supernatant were transferred into a new Eppendorf tube, 800 µl of ethanol added and the tubes were left at room temperature for 2 min. Samples were spun down for 3 min at full speed, the pellet was washed with 1 ml of 70% ethanol and spun for 3 min. The supernatant was taken off and the sample was dried. The dry pellet was resuspended in 30 µl of TE.

3.2.1.2. Isolation of Plasmid DNA from *Agrobacterium tumefaciens*

Same protocol used as for *E. coli*, see 3.2.1.1.

3.2.1.3. Isolation of Plasmid DNA from *Saccharomyces cerevisiae*

Yeast Miniprep Buffer:

2% (v/v)	Triton X-100
1% (w/v)	SDS
100 mM	NaCl
10 mM	Tris, pH 8
1 mM	EDTA

5 ml liquid cultures were grown in the appropriate selective medium for approx. 24 h. 1.5 ml of cells were transferred into a 1.5 ml Eppendorf tube and spun down in a benchtop centrifuge at full speed for about 1 min. The supernatant was decanted and the tubes were briefly mixed to resuspend the cells in the remaining liquid. 200 µl of yeast miniprep buffer and 200 µl of phenol:chloroform:isoamyl alcohol (25:24:1) were added and the solutions were mixed. 0.3 g of glass beads (approx. 150 µl of volume, glass beads previously sterilized by washing in nitric acid) were added and the tubes were mixed for 2 min. Samples were spun at full speed for 1 min. 150-180 µl were transferred into a new Eppendorf tube, and the DNA was precipitated by ethanol (see 3.2.5.1).

3.2.1.4. Isolation of Genomic DNA from *Arabidopsis thaliana*

Urea Extraction Buffer:

16.8 g	urea
5 g	Na_2SO_4
20 ml	1 M Tris pH 8
1.6 ml	0.5 M EDTA
1 g	N-lauroylsarcosine (sodium salt)

Dissolved in 100 ml of distilled H_2O and stored at 4° C.

5 x TE:

50 mM	Tris pH 8
5 mM	EDTA

3-4 *Arabidopsis* leaves were put in a 1.5 ml Eppendorf tube with 3 glass balls (2mm) and frozen in liquid nitrogen. Plant tissue was ground by using a vortex into fine powder and 500 µl of Urea Extraction Buffer were added. An equal volume of phenol/chloroform solution, equilibrated to pH 8, was added and mixed well. The samples were spun in a benchtop centrifuge at full speed for 10 min at 4° C. The aqueous phase was taken off and filtered through a piece of Miracloth. 400 µl of H_2O were added and the DNA was precipitated by adding 0.6 volume of isopropanol (480 µl). The sample was centrifuged at full speed for 10 min at 4° C. The DNA pellet was washed with 500 µl of 80% ethanol, spun at RT for 5 min at full speed and the pellet was dried. The DNA was resuspended in 500 µl 5x TE. 1/10 volume of 7.5 M NH_4Ac, pH 5.2 (50 µl) and 0.6 volume of isopropanol (350 µl) were added and the sample was spun at 4° C for 10 min at full speed. Washing of the DNA was repeated with 80% ethanol and the pellet was dried. The DNA was resuspended in 50 µl H_2O.

3.2.2. Isolation of RNA from *Arabidopsis thaliana* seedlings

Homo buffer:

330 mM	sorbitol
200 mM	Tris, pH 8
300 mM	NaCl
10 mM	EDTA
150 mM	β-mercaptoethanol

Wild-type seeds were surface sterilized, placed onto MS plates, vernalized for 2 days and moved into the growth room. Seedlings were harvested after 6 days in the growth room by removing all seedlings off the plates using forceps. The seedlings were ground in liquid nitrogen, immediately placed into 25 ml of a 1:1 solution of warm phenol (saturated in Homo buffer) and Homo buffer and homogenized for about 1 min. The samples were spun for 10 min in a benchtop centrifuge at 6 x g. The aqueous phase was removed and transferred into a new tube. Equal volume of chloroform:isoamylalcohol (24:1) was added, mixed and the samples were spun for 8 min at 6 x g. The aqueous phase was removed and distributed into new tubes. 1/3 volume of 8 M LiCl was added, mixed and the RNA was precipitated overnight at 4° C. The tubes were spun for 15 min at full speed, the supernatant was poured off and the pellet was washed with 1 ml of 70% ethanol. Again, the tubes were spun for 15 min at full speed and the supernatant was carefully removed. The pellet was resuspended in 200 µl H_2O and 600 µl EtOH together with 20 µl of 7.5 M NH_4Ac were added. Stored at -20° C.

3.2.3. Preparation of Protein Extracts

3.2.3.1. Preparation of a Protein Extract from *Arabidopsis thaliana*

Plant Protein Extraction Buffer:

50 mM	sodium phosphate buffer, pH 7
10 mM	EDTA, pH 7
0.1% (v/v)	Triton-X 100
0.1% (v/v)	sarkosyl (N-lauroylsarcosine)

After autoclaving, 10 mM β–mercaptoethanol were added.

Whole *Arabidopsis* seedlings were put into a 1.5 ml Eppendorf tube and frozen in liquid nitrogen. The tissue was ground by using a vortex and 50 µl Plant Extraction Buffer was added. The samples were spun in a benchtop centrifuge at 4° C for 10 min at full speed. The clear supernatant was taken off and transferred to a new Eppendorf tube. The extract was kept at 4° C.

3.2.3.2. Preparation of a Protein Extract from *Saccharomyces cerevisiae*

Lysis Buffer:

100 mM	Tris, pH 7.5
100 µM	EDTA, pH 8.0
5 mM	DTT
200 µM	PMSF

2 x Protein Sample Buffer:

4 ml	10% SDS
2 ml	glycerol
1.2 ml	1 M Tris pH 6.8
2.8 ml	H_2O
400 µl	β–mercaptoethanol

Method Yielding Stable Protein Extract:
1 - 1.5 ml of an overnight culture were put into a 1.5 ml Eppendorf tube and spun down at full speed in a benchtop centrifuge. The supernatant was discarded, the cell pellet resuspended in 100 µl of Lysis Buffer and the same volume of sterilized glass beads was added. The tube was mixed a total of 6 times for about 30 sec while placing sample on ice in between mixing steps. The sample was spun down at 3.5 x g for 10 - 15 min. The supernatant was transferred into a new Eppendorf tube (expected volume was around 60 µl) and about 20 µl of 2 x Protein Sample Buffer were added. The extract was boiled before using on protein gels.

Fast Extraction Method:
1 ml of overnight culture was spun down at full speed in a benchtop centrifuge. The supernatant was discarded and the cell pellet was resuspended in 50 µl of 2 x Protein Sample Buffer. The sample was frozen using liquid nitrogen or by putting the tube at -80° C. The extract was boiled for 5 min before use.

3.2.4. Protein and DNA Determination

3.2.4.1. Concentration of DNA, RNA or Oligonucleotides

To determine the concentration of nucleic acids within a solution, the optical density (OD) of the solution was measured at wavelengths of 260 nm, 280 nm and 320 nm after baselining the spectrometer against water. An OD reading of 1 at 260 nm corresponds to 50 µg of dsDNA, 40 µg of RNA or 33 µg of ssDNA.

3.2.4.2. Concentration of Proteins (Bradford)

Bradford reagent from Bio-Rad was prepared by diluting 1 part with 4 parts of distilled H_2O and filtered through a Whatman filter #1. A 1:10 dilution of BSA solution (10 µg/µl) was used to prepare standards of 2 µg, 4 µg, 6 µg, 8 µg and 10 µg of protein. 1 ml of diluted Bradford reagent was added to each of the standards and the samples to be measured. The tubes were briefly mixed and left at room temperature for about 10 min. The reaction was complete after about 5 min and stable for about an hour. The absorbance of samples was measured at 590 nm. A standard curve was created using the values for the known protein values and from this the protein concentration of the unknown samples was determined.

3.2.5. Enzymatic Reactions

3.2.5.1. Restriction Analysis of DNA

DNA restriction analysis was performed following the manufacturer's instructions. The enzyme concentration, digest temperature and buffer content of the reactions were chosen according to the recommendations by the manufacturer depending on the enzymes selected.
Depending on the further experimental design, a portion or the entire digest reaction was loaded onto an agarose gel (see 3.2.6.1) to visualize the result of the restriction digest. Fragments were then either purified out of agarose gels (see 3.2.6.1) or the DNA within the digest reaction was further purified by phenol-chloroform extraction and ethanol precipitation.
Lambda DNA (λDNA) restricted with *Bst*EII was used as molecular weight marker.

Phenol-Chloroform Extraction
The sample was mixed with an equal volume saturated phenol (in 1 M Tris, pH 8), thoroughly mixed and the organic and aqueous phases were separated by a centrifugation in a benchtop centrifuge at top speed. The upper aqueous phase was transferred into a fresh 1.5 ml Eppendorf tube and an equal volume chloroform:isoamyl alcohol (24:1) was added. Again, the tube was thoroughly mixed, the phases were separated by centrifugation and the aqueous phase was transferred into a fresh 1.5ml tube.

Ethanol Precipitation
To precipitate DNA out of solution, 2 1/2 times the sample volume of ethanol containing 300 mM NH_4Ac was added and the solutions were briefly mixed. The tube was incubated at -20° C for at least 1 h and the precipitated DNA was collected by centrifugation in a benchtop centrifuge at top speed. The supernatant was discarded and the pellet was washed once with 70% ethanol and then dried. The dried pellet was resuspended in various volumes of water or TE.

3.2.5.2. Dephosphorylation of DNA

For the reaction, the following components were added together:

20 µl	DNA (linearized vector)
10 µl	10 x CIP buffer (provided by enzyme manufacturer)
5 µl	CIP (Calf Intestinal Phosphatase, 10 U/µl)
65 µl	H_2O

Performed following the manufacturer's instructions and incubated at 37° C for approximately 1 h. 10 µl of 500 mM EDTA was added and the sample was incubated at 75° C for 10 min to inactivate phosphatase reaction. A phenol-chloroform extraction was performed followed by ethanol precipitation to purify the DNA.

3.2.5.3. Fill-in Reaction

To create blunt ends for further cloning, 5' protruding ends created by restriction reactions were filled by adding 10 mM dNTPs to a final concentration of 500 µM and 1 µl of Klenow DNA polymerase (2 U/µl). The sample was incubated for 10 - 15 min at 37° C followed by phenol-chloroform extraction and ethanol precipitation to purify DNA.

3.2.5.4. Ligation of DNA Fragments

Linearized vector (about 1 µg per reaction) was added to gel purified DNA insert in 1 x ligation buffer (provided by manufacturer). Optionally, the reaction also contained 100 µM ATP and 2% PEG. 1 - 3 µl of T4 ligase (30 U/µl) were added to the reaction with a total volume of 20µl. The ligation reaction was performed at room temperature overnight.

3.2.5.5. Reverse Transcription Reaction

Approximately 10 µg of *Arabidopsis* RNA (isolated from leafs or seedlings, RNA solution in ethanol) were precipitated by adding additional ethanol and spinning at full speed in a benchtop centrifuge for 20 min at 4° C. The RNA pellet was washed with 70% ethanol and dried. RNA was resuspended in 10 µl H_2O and 10 ng of oligo dT were added. The samples were put at 70° C for 5 min, then chilled on ice. The following was added:

1 µl	100 mM DTT
4 µl	5 x RT-buffer (provided by enzyme manufacturer)
2 µl	10 mM dNTPs
1 µl	RNasin (40 U/µl)
1 µl	AMV Reverse Transcriptase (25 U/µl)

Incubated at 42°C for 2 h. 1/10 of total volume was used as template for subsequent PCR reactions.

3.2.5.6. DNA Sequencing

Sequencing reactions were performed as described in Del Sal *et al.,* 1989, using the Sequenase polymerase from USB (Cleveland, OH).
DNA sequencing for some samples was performed at the Yale Medical School in the W.M. Keck Biotechnology Resource/HHMI Biopolymer Lab DNA Sequencing Group by PCR based sequencing using a ABI Prism 377 Genetic Analyzer.

3.2.5.7. Polymerase Chain Reaction

Polymerase Chain Reactions were performed using 1.25 units of Taq polymerase in a volume of 50 µl of 1 x Taq Polymerase buffer (supplied by the manufacturer). In addition to DNA template, the reactions contained 1.5 mM $MgCl_2$ and 200 µM of dNTPs. The primer concentration varied between 20 pmol and 2.5 µM. A typical reaction was heated at 85° C for 10 min and the denaturation step was performed for 1 min at 95° C. The annealing temperature was between 50° C and 65° C for 1 min depending on the primers used in the reaction. As final step, elongation was performed at 72° C for 1 min. Denaturation, annealing and elongation were repeated for 25 cycles unless noted otherwise.
PCR fragments for use as probes in Southern Blotting (see 3.2.8.1 and 3.2.5.8) were purified with QIAquick PCR Purification Kit from Qiagen following the instructions of the manufacturer.

3.2.5.8. Radioactive Labeling of DNA

Purified DNA probes (generated as described in 3.2.5.7) for Southern blotting (see 3.2.8.1) were labeled with [α-^{32}P] dCTP using the *redi*prime DNA labeling system from Amersham (Arlington Heights, IL) according to the manufacturer's protocol.

3.2.6. Gelelectrophoretic Methods

3.2.6.1. Separation of DNA and Isolation of Fragments

<u>Sequencing Gel</u>

Gel Solution for Sequencing (6%, 8M urea):

40 ml	5 x TBE
11.4 g	acrylamid
600 mg	bisacrylamid
96 g	urea

Adjusted to final volume of 200 ml with H_2O. Stirred slowly at RT to dissolve urea, filtered and stored at 4° C in a dark glass bottle. Reheated at 42° C before use to dissolve precipitated urea.

5 x TBE:

54 g	Tris
27.5 g	boric acid
4.65 g	EDTA (disodium salt)

Adjusted to final volume of 1 l with H_2O.

For a large gel, 70 ml of gel solution were degased and polymerized by adding 60 µl TEMED and 250 µl of freshly prepared 10% APS solution. The gel was prerun in 1 x TBE for at least 30 min at 35 W. Samples were loaded unto the gel and the gel was run at 35 W until the band of bromphenol blue contained in the sample reached the bottom of the gel.
The gel was carefully disassembled and put onto a 3MM Whatman paper and dried using a vacuum gel dryer. The dried gel was put into a film cassette and exposed to Kodak film.

Agarose gelelectrophoresis

Ethidium Bromide Stock Solution (1000 x):
500 µg/ml ethidium bromide in H_2O

Sample loading buffer (10 x):

20% (v/v)	glycerol
100 mM	Na_2EDTA, pH 8
1% (w/v)	SDS
0.25% (w/v)	bromphenol blue
0.25% (w/v)	xylene cyanol

50 x TAE:

242 g	Tris
57.1 ml	glacial acetic acid
37.2 g	EDTA

Adjusted volume to 1 l with bidest H_2O.

1 x TAE working solution:

40 mM	Tris acetate
2 mM	EDTA

Horizontal agarose gels were used to separate DNA fragments. Between 0.8 to 2% (w/v) agarose in 1 x TAE was brought to a boil. Ethidium bromide was added to a concentration of 1 x to the cooled solution before pouring the gel. Gel electrophoresis was performed in 1 x TAE with 1 x ethidium bromide added to the buffer until the desired separation of fragments was achieved. The separation was visualized under UV light and photographs of the gels were taken.

Isolation of DNA fragments
Specific fragments separated by electrophoresis were isolated from the agarose gel by using the QIAquick gel extraction kit from Qiagen according to the manufacturer's instructions.

3.2.6.2. Separation of Proteins and Coomassie Staining

Colloidal Coomassie Stock Solution:

1 g	Coomassie Brilliant Blue G-250
100 g	$(NH_4)_2SO_4$
20 g	H_3PO_4

Adjusted volume to 1 l. Shaken well before use.

Destain Solution:

10% (v/v)	glycerol

Gel Stock Solution (30%, 29:1):

58 g	acrylamide
2 g	bisacrylamide

Dissolved in 200 ml H_2O (bidest.) and filtered. Stored at 4° C in a dark glass bottle.

Lower Reservoir Gel Electrophoresis Buffer (10 x):

2 M	Tris pH 8.9

Upper Reservoir Gel Electrophoresis Buffer (10 x):

1 M	Tris pH 8.9
1 M	Tricine

To separate proteins, gel electrophoresis on polyacrylamid gel containing SDS (SDS-PAGE) was performed as originally described by Laemmli, 1970, using separating and stacking gels with different polyacrylamid and buffer concentrations as well as different pH values. Gels were run in a Bio-Rad Mini-Gel apparatus with 1 x gel electrophoresis buffer at a voltage of 100 V.
Additionally, 10% gels with the modifications described by Fling and Gregerson, 1986, were prepared as follows. For two running gel fitting the Bio-Rad minigel apparatus

4 ml	Gel Stock Solution (30%)
3 ml	3 M Tris pH 8.8
120 µl	10% SDS (w/v)
5 ml	H_2O

was added together and polymerisation was induced by adding 6 µl TEMED and 120 µl freshly prepared 10% APS solution. For the stacking gels

1 ml	Gel Stock Solution (30%)
750 µl	1 M Tris pH 6.8
60 µl	10% SDS (w/v)
4.25 ml	H_2O

were added together and polymerisation was induced by adding 4 µl TEMED and 60 µl freshly prepared 10% APS solution. Gels were placed into a Bio-Rad Mini-Gel apparatus with 1 x lower reservoir gel electrophoresis buffer in the lower buffer reservoir and 1 x upper reservoir gel electrophoresis buffer. 0.1% SDS were freshly added in the upper buffer reservoir and gels were run at 100 mA.
After completed electrophoresis, the separated proteins were either transferred onto nitrocellulose membrane (see 3.2.7.2.) or the protein bands were visualized by staining with Coomassie Brilliant Blue.
For staining, the required volume of solution was mixed with methanol at a 4:1 ratio. The gel was placed into the solution and stained with vigorous shaking to avoid settling of colloids. To achieve quantitative staining, the gel was kept in the Coomassie solution for 12 to 24 h. After completed staining, destaining of the gel background was performed in destain solution of 10% glycerol or in water.

3.2.7. Blotting of DNA and Proteins

3.2.7.1. Southern-Blot

20 x SSPE:

3.6 M	NaCl
20 mM	EDTA
200 mM	NaH_2PO_4

Upon separation of DNA fragments by agarose gel electrophoresis, a picture of the gel was taken while minimizing the exposure to UV light. The gel was put in a tray containing 0.4 N NaOH and incubated at room temperature for 30 min. The DNA fragments were blotted onto a nylon membrane by capillary action using paper towels and 3MM Whatman filters soaked in 400 mM NaOH. The transfer of DNA from the gel onto the membrane was performed for 2 h or o/n at room temperature.
The blotting setup was taken apart and the position of the gel wells was marked on the membrane. The membrane was put into a solution of 5 x SSPE.

3.2.7.2. Western-Blot

Blotting Buffer:

10 mM	CAPS, pH 11 (adjusted with NaOH)
10% (v/v)	methanol

Ponceau S staining solution:

0.2% (w/v) Ponceau S in 3% (v/v) acetic acid

After completed gel electrophoresis, the gel and the membrane were assembled in a Bio-Rad Trans-Blot SD Electrophoretic Transfer Cell between 5 layers of 3MM Whatman filters. The proteins were transferred onto the membrane by semi-dry electroblotting for about 30 min at 15 V.
The membrane was taken out of the blotting assembly and Ponceau S staining was performed to check the efficiency of the protein transfer onto the membrane. The membrane was added into a tray containing Ponceau S staining solution and staining was performed under shaking. Upon completion of staining, the membrane was rinsed in water and the position of protein marker bands was permanently marked onto the membrane using a ballpoint pen.

3.2.8. Visualization of Membrane-Bound Signals

3.2.8.1. Southern-Blot Hybridization

Hybridization Solution:

3 x	SSPE
10% (w/v)	SDS
0.2% (w/v)	PVP
0.2% (w/v)	Ficoll

Prehybridization Solution:

6 x	SSPE
30% (w/v)	SDS
0.2% (w/v)	PVP
0.2% (w/v)	Ficoll

SSD: 5 mg/ml solution of salmon sperm DNA

20 x SSPE:

3.6 M	NaCl
20 mM	EDTA
200 mM	NaH_2PO_4

Strip Solution:

0.1% (w/v)	SDS
0.1 x	SSPE

The membrane containing transferred DNA fragments (see 3.2.7.1.) was washed twice in 5 x SSPE for 10 min each. The membrane was dried briefly between 2 layers of Whatman filter, rolled up and put into a hybridization bottle. Salmon sperm DNA (SSD) was denatured at 95° C for 10 min and immediately snap cooled on ice. 50 ml of prehybridization solution and 500 µl of the denatured SSD were added into the hybridization bottle containing the nylon membrane. Prehybridization was performed at a temperature of 65° C for 2 - 3 h.
The prehybridization solution was replaced by 10 ml of hybridization solution containing 100 µl of SSD. The appropriate DNA probe and 1 kb ladder that had been labeled following 3.2.5.8. were denatured at 95° C for 10 min, immediately snap cooled on ice and added to the hybridization bottle. Hybridization was performed at 65° C for 2-3 h or o/n.

The membrane was washed twice with 50 ml of wash solution for about 30 min each. The membrane was removed, briefly dried between two layers of Whatman filters and placed into a plastic bag. The radioactive signals on the membrane were visualized on Kodak film.

3.2.8.2. Immunostaining of Filter-Bound Protein

Blocking Solution:

1 x	TTBS
5% (w/v)	Carnation dry milk powder

10 x TBS:

500 mM	Tris pH 7.5
1.5 M	NaCl

TTBS:

1 x	TBS
0.05%	Tween 20

After electrophoresis and transfer of the separated proteins onto a nitrocellulose membrane (see 3.2.6.2 and 3.2.7.2), the membrane was put into blocking solution and incubated under slow shaking o/n at 4° C. The membrane was rinsed twice in TTBS and then transferred into a solution of 3% BSA in TTBS with 1:1500 diluted anti-HA-antibody from mouse and incubated for 1 h at room temperature. Following that, the membrane was transferred into blocking solution with a 1:3333 dilution of anti-mouse IgG_{2b} peroxidase conjugate and incubated for 1 h at room temperature. Alternatively, an incubation of 2 h at room temperature was performed with a 1:5000 dilution of anti-PP2A C antibody from rabbit in TTBS followed by an incubation with a 1:5000 dilution of anti-rabbit antibody with peroxidase conjugate in blocking solution for 1 h at room temperature. Following both antibody combinations, the membrane was washed once for 5 min in blocking solution, twice for 5 min in TTBS and twice for 5 min in 1 x TBS. The membrane was briefly dried between 2 sheets of 3MM Whatman paper and placed into a plastic bag.
In the darkroom, LumiGlo chemiluminescence substrate solutions were mixed according to the manufacturer's instructions and 2 ml were added into the plastic bag containing the membrane and incubated for 1 min in the dark. The LumiGlo solutions were removed and the membrane was put onto an undeveloped piece of Kodak film for various timepoints between 1 sec to 1 min.

3.2.9. Transformation Methods

3.2.9.1. Bacteria

3.2.9.1.1. Preparation of Electrocompetent *Escherichia coli* Cells

EC buffer:

15% (v/v)	glycerol
1 mM	HEPES, pH 8

Using EC buffer:
An *E. coli* strain was streaked onto a LB plate containing an appropriate antibiotic and was incubated at 37° C o/n. 50 ml liquid media culture (LB with appropriate antibiotic) were inoculated using a fresh single colony and incubated in a water shaker at 37° C o/n. 400 µl of overnight culture were used to inoculate 400 ml liquid culture (LB with appropriate antibiotic) and incubated in a water shaker at 37° C. The OD reading at 600 nm was checked at regular intervals. After the OD_{600} reading reached 0.3, the culture flask was moved into the coldroom and put on ice for 20 min. All subsequent steps were performed on ice. The cells were spun down at approx. 1900 x g for 10 min and the supernatant was removed. The cell pellet was washed in 40 ml of EC buffer and spun down again. Cells were resuspended in 4 ml of EC buffer and stored 100 µl aliquots at -80° C.

Using 10% glycerol solution (protocol from Bio-Rad):
An *E. coli* strain was streaked onto a LB plate containing an appropriate antibiotic and was incubated at 37° C o/n. 50 ml liquid media culture (LB with appropriate antibiotic) were inoculated using a fresh single colony and incubated in a water shaker at 37° C o/n. The entire overnight culture was used to inoculate a 500 ml liquid culture (LB with appropriate antibiotic) which was incubated in a water shaker at 37° C. The OD reading at 600 nm was checked at regular intervals. After the OD_{600} reading reached a value between 0.5 and 0.8, the culture flask was chilled on ice in the coldroom for 15 to 30 min. Cells were spun down at approx. 1900 x g for 15 min and the supernatant was removed. The cell pellets were washed once in 250 ml of cold, autoclaved H_2O and spun down again. The cell pellets were combined in a total volume of 250 ml of cold, autoclaved H_2O and spun down. Cells were resuspended in approx. 10 ml of 10% glycerol (v/v) and spun down. Finally, the cells were resuspended in about 1 ml of 10% glycerol (v/v) and stored 40 µl aliquots at -80° C.

3.2.9.1.2. Preparation of Electrocompetent *Agrobacterium tumefaciens* Cells

EC buffer:

15%	glycerol
1 mM	HEPES, pH 8

Agrobacteria GV3101 were streaked on a LB-Strep plate and incubated at 28° C for 2 days. A 50 ml liquid LB-Strep culture was inoculated with one colony from plate and grown o/n at 28° C with vigorous shaking. A 200ml liquid LB-Strep culture was inoculated by adding the whole overnight culture to the new media and was incubated at 28° C with vigorous shaking. The OD reading at 600 nm was checked at regular intervals. After the OD_{600} reading reached a value between 0.3 and 0.5, the culture flask was taken out of the incubator and put in the coldroom on ice for 20 min. The cells were spun down at approx. 3000 x g for 10 min and the supernatant was discarded. Cells were washed twice with 40 ml of EC buffer and centrifuged at approx. 3000 x g for 10 min. Finally, the bacterial pellet was resuspended in 2 ml of EC buffer and stored in 150 µl aliquots at -80° C.
(All materials in contact with agrobacteria were soaked in a bleach solution o/n and autoclaved for a total of 80 min to avoid contamination in the laboratory.)

3.2.9.1.3. Bacterial Transformation (Electroporation)

The cuvettes and sliding cuvette holder were chilled at -20° C for several minutes before electroporation. Electrocompetent cells were thawed on ice. DNA was added to cells, mixed gently and let sit on ice for about 1 min. The mixture of cells and DNA was transferred into a cold cuvette, ensuring that sample was at the bottom of the cuvette. The outside of the cuvette was wiped dry and the cuvette was placed into a Bio-Rad Gene Pulser apparatus. One pulse was applied at the settings of 25 µF capacitor, 2.5 kV and 200 Ω pulse controller. Immediately 1 ml of LB was added, the mixture was placed into 25 ml culture tube and incubated at 37° C for 1 h. The cells were transferred into a 1.5 ml Eppendorf tube and spun down. The supernatant was removed and the cells were spread onto media plates containing appropriate antibiotics for selection.

3.2.9.2. Yeast

3.2.9.2.1. Preparation of Electrocompetent *Saccharomyces cerevisiae* Cells

10 x TE, pH 7.5:
100 mM Tris
10 mM EDTA
Adjusted pH with conc. HCl to 7.5. Autoclaved.

10 x LiAc, pH 7.5:
1 M LiAc
Adjusted pH to 7.5 with acetic acid. Autoclaved.

TE: 10 mM Tris, pH 8
1 mM EDTA
Autoclaved.

Preparation of cells for high efficiency transformation (Schiestl and Gietz, 1989)
This method was modified from Schiestl and Gietz, 1989. Overnight cultures were grown in 5 ml liquid media (YPD or appropriate selection media) at 30° C. OD_{600} measurement of cultures were taken and a 200 ml liquid culture was inoculated to an OD_{600} = 0.1. The 200 ml culture was grown at 30° C to an OD_{600} reading of 0.4 - 0.5. The cells were spun down at approx. 1900 x g for 5-10 min. Cells were washed in 10 ml of sterile H_2O and centrifuged again at 1900 x g for 5-10 min. A second wash was performed using 1 ml of sterile H_2O, the cells were transferred into a 1.5 ml tube and spun at 4.8 x g for 1 min. The cells were resuspended in 1 ml of 1 x TE, 1 x LiAc (freshly prepared from 10 x stock solutions) and incubated at 30° C for 1 h with slow agitation. Used immediately for transformation.

Preparation of cells for large scale transformation (modified from: MATCHMAKER Two-Hybrid System, Clontech Laboratories)
An overnight culture was grown in 5 ml liquid medium (YPD or appropriate selective media). The entire overnight culture was used to inoculate a 50 ml culture of the same media and was grown overnight. An OD_{600} reading was taken and a 300 ml culture of the same medium was inoculated to an OD_{600} of 0.2-0.3. The culture was grown until the cells had doubled and was then spun down at room temperature for 5 min at 3000 rpm using a GSA rotor. The supernatant was discarded, the cells were washed in a total volume of 25 ml TE and spun down for 5 min at 3000 rpm using a SS34 rotor. The supernatant was again discarded, the cells were resuspended in 1.5 ml of 1 x TE, 1 x LiAc (freshly prepared from 10 x stock solutions) and immediately used for transformation.

3.2.9.2.2. Yeast Transformation

50% PEG 4000:
Dissolved 50 g of PEG 3350 in 100 ml autoclaved H_2O.

SSD: 10 mg/ml solution of salmon sperm DNA
(sonicated to sheer DNA and reduce size of fragments)

High efficiency transformation (Schiestl and Gietz, 1989)
This method was modified from Schiestl and Gietz, 1989, using freshly prepared competent yeast cells (see 3.2.9.2.1.). Plasmid DNA (up to 5 µg) was prepared in a final volume of 5 µl. Salmon sperm DNA (SSD) was boiled for 10 min, chilled on ice and 15 µl of carrier DNA was mixed with plasmid DNA. 200 µl of yeast cell suspension was added to 1 ml of freshly prepared 40% PEG 4000, 1 x TE, 1 x LiAC and incubated for 30 min at 30° C with slow agitation. The cells were heat shocked for 15 min at 42° C and spun down for 1 min at 4.8 x g. The cells were washed twice in 1 ml of 1 x TE, carefully resuspended using a pipette and spun down at 4.8 x g for 1 min. The cells were resuspended in 0.5 - 1 ml of 1 x TE and 100 - 200 µl of cells were plated on appropriate selective medium. Plates were incubated at 30° C.

Large scale transformation (modified from: MATCHMAKER Two-Hybrid System, Clontech Laboratories)
About 20 µg of plasmid DNA (i.e. library DNA) were prepared and mixed with 2 mg of SSD. 1 ml of freshly prepared competent cells (see 3.2.9.2.1) were added and mixed well. A solution of 1 x TE, 1 x LiAc, 40% PEG was freshly prepared and 6 ml were added to the mixture of DNA and cells. The mixture was briefly mixed and incubated at 30° C for 30 min under slight rotation. 700 µl of DMSO was added, mixed by inversion and the cells were heat shocked at 42° C for 15 min while mixed occasionally. The cells were chilled on ice for 2 min and then spun down for 5 min at 3000 rpm using a SS34 rotor. The supernatant was carefully removed by pipetting and the cells were resuspended in 2 x 25 ml of media selecting for the transformed plasmid. The cells were incubated at 30° C for 1 h with constant agitation. The cell suspension was again spun down 5 min at 3000 rpm using a SS34 rotor, the supernatant carefully removed and the cell pellet resuspended in TE buffer to a total volume to about 7.5 ml. Cells were plated onto appropriate selective medium and the plates were incubated at 30° C.

3.2.9.3. Plants

3.2.9.3.1. Growing and Preparation of *Arabidopsis thaliana* for Transformation

Wet soil was placed into plastic cups, covered with a piece of screen material and the screen was fixed into position using rubber bands. Seeds were suspended in water and distributed onto the soil using a syringe. The cups were put into the plant room. Each cup should have 6 - 8 plants for transformation, therefore extraneous seedlings were removed after germination. Once the primary inflorescences reached a height of 2 - 3 cm (after approx. 6 weeks), the bolts were cut to induce growth of multiple secondary inflorescences and plants were used for transformation 4 to 8 days after clipping. Before vacuum infiltration, all opened flowers were cut leaving only unopened bolts.

3.2.9.3.2. Plant Transformation (Vacuum Infiltration)

Infiltration Medium:

1/2 x	Murashige & Skoog salts (with B5 vitamins)
5% (w/v)	sucrose
44 nM	benzylamino purine (1 mg/ml stock in DMSO)

The protocol used is based on Bechtold *et al.*, 1993 with modification by A. Bent as published on the *Arabidopsis* network (http://genome-www.stanford.edu/cgi-bin/biosci_arabidopsis).
Large liquid cultures of *Agrobacteria* carrying the desired constructs were grown at 28° C under vigorous shaking for about 2 days until the bacterial cultures reach an $OD_{600} \geq 0.8$. Cells were spun down, the medium discarded and the cells resuspended in infiltration medium using 1/3 of the original volume.
200 ml of agrobacterial solution were filled into a beaker, a plant pot was inverted into the solution and secured into place and placed into a bell jar. Vacuum was applied until bubbles formed on leaf and stem surface of the plants and the agrobacterial solution started to bubble. The application of vacuum was repeated a total of 3 x 2 min for each pot of plants. Upon releasing the vacuum, the plants were removed and the pots were wrapped with saran wrap. The plants were moved into the plant room and laid on their sides for 24 h. Plants were unwrapped, set upright and grown while ensuring that plants carrying different constructs were kept separate. When siliques on plants were very dry, seeds were harvested and tested on selection plates (see 3.2.10.3.).

3.2.9.3.3. Isolation of Protoplasts from *Arabidopsis thaliana*

Enzyme Solution:

0.25% (w/v)	macerozyme
1% (w/v)	cellulase
30 mM	$CaCl_2$
5 mM	β-mercaptoethanol
0.1% (w/v)	bovine serum albumin
10 mM	MES-KOH, pH 5.6

in 500 mM mannitol solution.

MaMg Solution:

400 mM	mannitol
15 mM	$MgCl_2$
5 mM	MES-KOH, pH 5.6

W5 Solution:

154 mM	NaCl
125 mM	$CaCl_2$
5 mM	KCl
5 mM	glucose
1.5 mM	MES-KOH, pH 5.6

Wash Solution:

2 volumes	500 mM mannitol solution
1 volume	200 mM $CaCl_2$

The protocol was modified from Abel and Theologis, 1994. Leaves of approximately 5 weeks old plants were chopped using fresh, sharp razor blades and immediately put into a 500 mM mannitol solution. Cut leaves were filtered out of the mannitol solution and placed into an appropriate amount of freshly prepared enzyme solution, so that the leaves were completely covered. Vacuum was applied to the solution for about 20-30 sec followed by an incubation for 3 h in the dark with gentle shaking. An equal volume of 200 mM $CaCl_2$ was added and the solution was filtered through nylon mesh to remove plant material. The isolated protoplasts were spun down for 5 min at 60 x g and the supernatant was carefully removed. The pellet was resuspended in 3-4 ml of 600 mM sucrose solution and carefully layered on top of 600 mM sucrose solution to reach a final volume of 25 ml. The tubes were spun for 5 min at 40 x g. Protoplasts in the upper phase were collected by carefully removing plant debris pellet and solution underneath the protoplast layer. 30 ml of Wash Solution were added and the tubes spun for 5 min at 40 x g. Protoplast pellets were resuspended in 5 - 10 ml of W5 buffer and incubated on ice for at least 30 min. The protoplasts were spun down and an aliquot was counted under the microscope using a hematocytometer. The cell pellet was resuspended to a final protoplasts concentration of at least 1 x 10^6/ml.

3.2.9.3.4. Transformation of Protoplasts

Incubation Medium:

500 mM	mannitol
5 mM	KCl
4 mM	MES-KOH, pH 5.6
5 mM	$CaCl_2$

PEG-CMS Solution:

400 mM	mannitol
100 mM	$Ca(NO_3)_2$
40% (w/v)	PEG 6000

W5 Solution:

154 mM	NaCl
125 mM	$CaCl_2$
5 mM	KCl
5 mM	glucose
1.5 mM	MES-KOH, pH 5.6

The transformation protocol was modified from Abel and Theologis, 1994. 300 µl of the protoplast solution (see 3.2.9.3.3.) were put into a 15 ml polypropylene tube, 10 µl of a 5 mg/ml salmon sperm stock solution were added and the solutions mixed carefully. 50 µg of plasmid DNA were added, mixed carefully and incubated at RT for 5 min. An equal volume of PEG-CMS solution was added, mixed carefully to achieve a homogeneous solution and incubated at RT for 30 min. 4 dilution steps were performed by adding 600 µl, 1 ml, 2 ml and 4 ml of W5 solution and mixing carefully after each addition. The tubes were spun for 5 min at 60 x g, the supernatant was removed and the pellet washed once in a 4:1 solution of 400 mM mannitol/W5 solution. The protoplast pellet was resuspended in 1 ml of incubation medium and filled into small petri dishes that had previously been coated with approximately 2-3 ml 1:5 diluted FBS (fetal bovine serum) for about 10 min. Protoplasts were incubated for 24 h in constant white light. GUS activity was determined as described in 3.2.11.3.

3.2.10. Growing of *Arabidopsis thaliana* Plants

3.2.10.1. Growth Conditions

Arabidopsis thaliana (L.) Heynh., ecotype Columbia plants were grown in a plant culture room at 22° C, 60% relative humidity under 16 h white light (90 µE m^{-2} sec^{-1}) and 8 h dark day cycles unless noted otherwise.
Light experiments with transgenic plants were performed under continuous light with quantum measurements as follows: White light at 110 µE m^{-2} sec^{-1}, red light at 31 µE m^{-2} sec^{-1}, far-red light at 175 µE m^{-2} sec^{-1} and blue light at 50 µE m^{-2} sec^{-1}.

3.2.10.2. Surface Sterilization of Seeds

Surface sterilization solution:

25% (v/v)	Chlorox bleach
0.1% (w/v)	SDS

Seeds were filled into 1.5 ml Eppendorf tubes (approx. 75 - 100 µl of volume, 100 µl of seed volume equal approximately 3000 seeds) and 1.2 - 1.4 ml of surface sterilization solution was added. The tubes were gently shaken for about 10 - 15 min. After seeds settled, the supernatant was taken off and the seeds were washed by adding 1 ml of sterile H_2O. The water was taken off after seeds had settled again and the wash step was repeated for a total of 4 - 5 times. Seeds were suspended in 0.1% agar and put on appropriate media plates. The plates were vernalized for 48 h at 4° C and then subjected to different growth conditions.

3.2.10.3. Segregation Analysis

Sterilized seeds (see 3.2.10.2) from vacuum transformed plants were placed onto selection plates (3.1.5.3.), the plates were vernalized for 48 h at 4° C and placed into the plant room. Plantlets showing hygromycin resistance were transferred onto MS plates containing no antibiotic with 0.8% agar to promote root growth (rooting plates, see 3.1.5.3.). Plants were transferred onto soil, grown and the seeds were harvested. 50 seeds each of the F1 generation were then placed onto selection plates again, and the number of surviving seedlings was recorded. Four of the surviving seedlings were transferred into soil, plants were grown and the seeds harvested. The same process was repeated for the third generation of transgenic seedlings after transformation. Lines that showed the expected segregation rate of 3:1 for the second generation and 100% for the third generation were selected.

3.2.10.4. Crossing of *Arabidopsis thaliana* Plants

Unopened flowers were selected on the female parent and all other flowers were removed. Everything but the ovary was removed from the flowers. As male parent ColWT plants were selected and older flowers were used. Under the microscope, flowers with yellow anthers were carefully removed by forceps and used to place pollen on the stigma of the female plant. A total of 5 - 6 flowers of each female parent plant were pollinated in this manner and labeled by carefully placing white thread around the stem. Once dried siliques were formed, they were carefully removed and the seeds harvested.

3.2.11. β-Glucuronidase (GUS) Analysis

3.2.11.1. GUS Staining

Clearing Agent:

½ vol.	chloral hydrate
¼ vol.	lactic acid
¼ vol.	phenol

Warmed up to about 60° C for a few minutes and stirred to dissolve. Stored in a dark glass bottle at RT.

GUS Staining Solution:

100 mM	sodium phosphate buffer, pH 7.0
10 mM	EDTA, pH 7.0
2 mM	K-Ferrocyanide, pH 7.0
2 mM	K-Ferrocyanide, pH 7.0
1 mM	X-glucuronide
0.1% (v/v)	Triton-X 100
2/10 vol.	methanol

The staining procedure was modified from Stomp, 1992 and the clearing agent was used as described in Beeckman and Engler, 1994.
Seedlings grown under different light conditions were harvested and submerged into GUS staining solution. Vacuum was applied for 5 min and the samples were then incubated at 37° C in the dark. Different timepoints were taken at 1 h, 2 h, 4 h, 8 h and 24 h by transferring seedlings into fixation solution of 3:1 ethanol:acetic acid. Tissues were fixed for 30 - 60 min. Seedlings were transferred into 70% ethanol and could be stored at 4° C. Seedlings were put onto microscope glass slides and the staining was examined under the microscope. Alternatively, the seedlings were taken out of the GUS staining solution at different timepoints and put directly onto microscope glass slides. 40 - 50 µl of clearing agent was added, the slide was incubated o/n at RT and the stained samples were then examined under the microscope.

3.2.11.2. GUS Activity

Fluorimetric MUG Assay Buffer:

35 mg 4-methyl umbelliferyl glucuronide (MUG) in 100 ml Plant Protein Extraction Buffer (see 3.2.3.1). Stored in the dark at 4° C.

The fluorometric assay was performed in modified form based on Jefferson *et al*., 1987. Plant protein extracts (see 3.2.3.1.) were prepared from seedlings grown under different light conditions. 50 µl of protein extract were used and added to 450 µl Fluorimetric MUG Assay Buffer. 100 µl were transferred into a new tube and samples were incubated at 37° C in the dark for different periods of time. The reaction was stopped by adding 900 µl of 200 mM Na_2CO_3 and the samples were kept in the dark until measurement. The fluorescence reading was determined using a Digital Fluorometer (Sequoia-Turner Corporation, model 450) at an excitation wavelength of 365 nm and an emission wavelength of 455 nm. The GUS activity was calculated using fluorescence readings of known standards of 4-methyl-umbelliferone (4-MU) in 200 mM

Na_2CO_3 (25 nM, 50 nM, 75 nM, 100 nM, 200 nM, 500 nM, 750 nM, 1 mM and 2 mM). The protein concentration of the extracts was determined (see 3.2.4.2.).

3.2.11.3. GUS Assay on Transformed Protoplasts from *Arabidopsis thaliana*

GUS Assay Buffer:

50 mM	Na_2HPO_4
0.1% (v/v)	Triton-X 100
200 µM	PMSF (200 mM stock solution in isopropanol)

Previously incubated protoplasts were collected from plates into 1.5 ml Eppendorf tubes, spun down at low speed and the supernatant was removed (pellets can be frozen at that point). The pellet was resuspended in 300 µl of GUS assay buffer, mixed, the samples frozen at -80 °C, thawed and mixed again. The tubes were spun at high speed for 10 min and the supernatant was transferred into a new 1.5 ml tube. 50 µl of protoplast extract were used, added to 150 µl of 50 mM Na_2HPO_4 and 2 µl of 100 mM 4-MUG and incubated in the dark at 37 °C. Time points were taken at 30 min, 60 min and 120 min or duplicate time points at 60 min by removing sample from the 37 °C incubation and adding 1 ml of 200 mM Na_2CO_3 to stop the reaction. Samples were kept in the dark until measurement. The fluorescence of the samples was determined using a Digital Fluorometer (Sequoia-Turner Corporation, model 450) at an excitation wavelength of 365 nm and an emission wavelength of 455 nm. The GUS activity was calculated using fluorescence readings of known standards of 4-methyl-umbelliferone (4-MU) in 200 mM Na_2CO_3 (25 nM, 50 nM, 75 nM, 100 nM, 200 nM, 500 nM, 750 nM, 1 mM and 2 mM). The protein concentration of the extracts was determined (see 3.2.4.2.).

3.2.12. Whole-Mount *In situ* Hybridization

3.2.12.1. Probe Labeling

HEMA1 cDNA was used as a probe for the *in situ* hybridization. The plasmid pAH6 containing a cDNA clone of *HEMA1* in a pBluescript II SK vector background was obtained as a gift from Dr. A. Madan Kumar (Fig. 6). The *HEMA1* cDNA was flanked by a T7 promoter at the 3' end, whereas a T3 promoter was available on the 5' end. Therefore, both sense and antisense transcripts were possible using the appropriate polymerase. Restriction enzymes cutting 5' and 3' were selected to linearize the vector containing the *HEMA1* cDNA to allow for run-off transcription generating antisense and sense transcripts, respectively. To avoid transcripts of the wrong strand through run-on transcription by the polymerase using the 3' end as a substrate, restriction enzymes that produce 5' overhangs were used. During *in vitro* transcription, digoxigenin (DIG) labeled UTP was used to created DIG-labeled RNA transcripts that can be detected by anti-DIG antibodies after hybridization.

Linearization of Plasmid

Restriction analysis using appropriate restriction enzymes was performed to double-check the orientation of the *HEMA1* insert (Fig. 6). A restriction digest (see 3.2.5.1) using *Not*I was performed to linearize the plasmid for *in vitro* transcription utilizing the T7 promoter resulting in the antisense transcript. A digest using *Sal*I was performed to linearize the plasmid for T3 transcription creating the sense transcript which was used as negative control. About 10 µg of plasmid DNA each were used for both digests in a total volume of 100 µl and the restriction digest reactions were incubated at 37° C for 5 1/2 h. 0.5 µl of each digest were used to confirm the completion of the digest reaction on an agarose gel. The linearized plasmid DNA was the isolated from both reactions by performing phenol-chloroform extraction and ethanol precipitation (see 3.2.5.1). The resulting DNA pellets were resuspended in 9 µl of water.

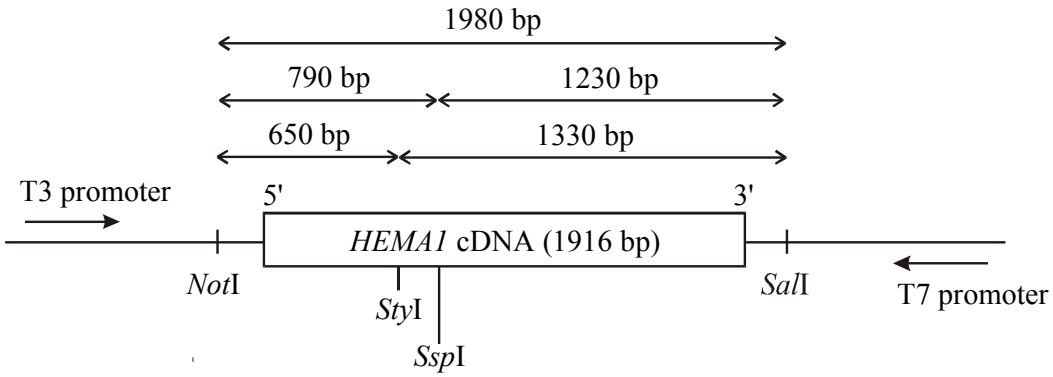


Figure 6: Vector map of pAH6 containing the *Arabidopsis HEMA1* cDNA.

In vitro Transcription

10 x txn buffer +:

200 mM	Tris pH 7.9
200 mM	$MgCl_2$
20 mM	spermidine

The linearized plasmid DNA was used to set up *in vitro* transcription reactions as follows. In these reactions, the plasmid pAH6 cut with *Not*I was used with T7 polymerase to create DIG-labeled antisense RNA. The same plasmid DNA cut with *Sal*I pAH6 was used with T3 polymerase to create DIG-labeled sense RNA transcript.

1 µl	linearized plasmid DNA (about 1 µg)
2.25 µl	10 x txn buffer +
1 µl	100 mM DTT
1 µl	10 x BSA (1 mg/ml)
2 µl	10 x DIG RNA labeling mix (supplied by Roche Boehringer Mannheim)
2 µl	GMP (100 mM)
1 µl	RNasin (40 U/µl)
7.5 µl	H_2O
2 µl	T3 RNA Polymerase (20 U/µl) or T7 RNA Polymerase (gift from Dr. M. Ibba)

The *in vitro* transcription reactions were incubated at 37° C for 4 h. Both reactions were brought to a final volume of 100 µl by adding water.

DNAse Digestion

To 50 µl of the transcription reaction, the following was added:

10 µl	10 x DNAse buffer (provided by enzyme manufacturer)
38 µl	dH_2O
2 µl	DNAse I (Roche Boehringer Mannheim, 10 mg/µl)

The reactions were incubated at 37° C for about 1 h and then the following was added to each reaction:

4 µl	500 mM EDTA
6.5 µl	8 M LiCl
300 µl	ethanol

The DNA was precipitated by storing both reaction tubes at -80° C overnight. DNA was spun down for 30 min at maximum speed in a benchtop centrifuge and the isolated DNA pellets were air dried.

Hydrolysis

50% formamide, RNasin solution:

200 µl	formamide
199 µl	H_2O
1 µl	RNasin (Promega, 40 U/µl)

In this step, the labeled riboprobes are digested to an average length of 150 nt by controlled alkaline hydrolysis as described in Cox *et al.*, 1984. The required time of hydrolysis to achieve fragments of desired length, was calculated using the following formula:

$$\text{hydrolysis time} = \frac{\text{(initial transcript length in kb) - (desired length in kb)}}{\text{k (initial transcript length in kb) (desired length in kb)}}$$

with the constant "k" being approximately 0.11 cuts/kb/min.

As initial transcript length was 1.9 kb and the desired length of probes was 150 bp, the necessary hydrolysis time was calculated as 58 min.

The DNA pellets from the previous DNAse digests were dissolved in 50 µl of 100 mM NaHCO3 and incubated at 60 ° C for 58 min. Then

5 µl	5% acetic acid
5.5 µl	3 M NaAc
180 µl	EtOH

were added and the DNA was precipitated at -80° C overnight. Both reactions were spun down for 30 min at maximum speed in a benchtop centrifuge and the DNA pellets were air dried.
The DNA was resuspended in 25 µl of 50% formamide, RNasin solution. Agarose gel electrophoresis was performed using a 0.8% agarose gel with fresh gel buffer to prevent RNA degradation. 3 µl of both samples were compared to samples from the original *in vitro* transcription reactions to check completion of the DNAse and hydrolysis reactions and against a known amount of tRNA to obtain an estimated RNA concentration.
For the use in the *in situ* hybridization experiment, a RNA concentration of 10 ng/µl for low abundant messages is recommended with the final volume being 200 µl. Therefore a total of about 2 µg of RNA was used for both the sense and antisense probe. To stabilize the RNA probes, 20 µg of salmon sperm DNA was added within the 200 µl of probe volume to a final concentration of 0.1 mg/ml.

3.2.12.2. Preparation of Plant Seedlings

A. thaliana (ecotype: Columbia) wild-type seeds were plated onto 0.8% agar plates containing 1/2 MS media with 1% sucrose and vernalized in the coldroom at 4° C for one week (see 3.1.5.2, 3.2.10.2.). The plates were moved into the plant room and half the plates were grown under normal light conditions for 5 days (see 3.2.10.1.). The other half was given a 5 hour light treatment after which the plates were wrapped in aluminum foil and grown in the dark for 5 days. The seedlings were harvested and used for *in situ* hybridization.

3.2.12.3. *In situ* Hybridization

BS: PBT with

4% (w/v)	BSA fraction V
0.1% (v/v)	Tween 20

FB (pH 7.4):

PBT with	
80 mM	EGTA
5% (v/v)	formaldehyde
10% (v/v)	DMSO

HS:	50% (v/v)	formamide
	5 x	SSPE
	50 µg/ml	heparin

NTE:	500 mM	NaCl
	10 mM	Tris, pH 7.5
	1 mM	EDTA

PBT (pH 7.4):

137 mM	NaCl
3 mM	KCl
10 mM	Na_2HPO_4
5 mM	KH_2PO_4

After autoclaving, 0.1% (v/v) Tween 20 was added.

SB:	100 mM	NaCl
	50 mM	$MgCl_2$
	100 mM	Tris, pH 9.5
	0.1% (v/v)	Tween 20

SSB:	PBT with	
	20 mM	EDTA

20 x SSPE:

3.6 M	NaCl
20 mM	EDTA
200 mM	NaH_2PO_4

In situ hybridization was performed following a modified protocol described by de Almeida Engler *et al.*, 1994, with a 2% driselase digestion step added to the prehybridization treatment.

Fixation

Both light grown and dark grown seedlings (see 3.2.12.2) were carefully removed from the media plates, placed into glass scintillation vials filled with FB buffer and the fixation was performed under vacuum for 30 min.

Dehydration

Samples were dehydrated by washing them 2 times for 5 min in 100% methanol and 4 times for 5 min in 100% ethanol while shaking gently.

Prehybridization

All prehybridization steps were performed with the glass vials gently shaking. Samples were rinsed twice for 5 min in 100% ethanol and then washed for 30 min in a 1:1 ethanol:xylene mixture. Samples were then washed twice for 5 min in 100% ethanol and then twice for 5 min in 100% methanol. After that, a washing step in a 1:1 methanol:PBT mixture for 10 min was performed. The samples were post-fixed by placing them into PBT with 5% formaldehyde for 30 min and then rinsed three times for 1 min in PBT. PBT with 2% driselase and 0.2% pectolyase was then introduced into the tissue under vacuum for about 20 to 25 min followed by two rinses for 1 min with PBT. To further prepare for efficient probe penetration, the samples were digested for 13 min in PBT with 40 µg/ml proteinase K. The digest was stopped by two 5 min washes in PBT with 0.2% glycine followed by two 1 min rinses in PBT. Samples were further post-fixed by placing them into PBT with 5% formaldehyde and then rinsed three times in PBT for 1 min. To prepare for

hybridization, samples were washed for 5 min in a 1:1 PBT:HS mixture, rinsed twice for 1 min in HS and then pre-hybridized for 1 - 2 h at 55 - 60° C in HS.

Hybridization

Plant samples were transferred into 1.5 ml Eppendorf tubes. 200 µl of both prepared antisense and sense probes (see 3.2.12.1) were denatured for 3 min at 100° C and then added to light and dark grown seedlings. Hybridization was performed at 56° C for 15 h under gentle shaking.

Washing

The hybridization mix was removed and the plant samples were transferred into cell tissue plates which were used for all further hybridization wash steps. The plant samples were washed twice in pre-warmed HS at 50° C for 30 min followed by a 60 min wash in a 1:1 mixture of HS and NTE at room temperature. After that the samples were washed for another 60 min in NTE and rinsed for 1 min with NTE at room temperature. The plates were transferred to 37° C and the samples were incubated in pre-warmed NTE containing 40 µg/ml RNAse A for 45 min. This was followed by a wash with pre-warmed NTE for 15 min at 37° C after which the plates were transferred back to room temperature. The samples were washed four times for 15 min in NTE and were then equilibrated during a 15 min wash in a 1:1 mixture of NTE and PBT. After a 1 min rinse in PBT, the samples were incubated for 30 min in BS.

Preabsorption of Anti-DIG Antibody Conjugate to Plant Tissue

30 mg of plant powder (gift from Jean Deruère) were heated at 100° C for 10 min and then dissolved in 800 µl of PBT with 2% BSA. 20 µl of anti-DIG Fab fragment alkaline phosphatase conjugate (Roche Boehringer Mannheim) were added and the mixture was incubated overnight at room temperature. To clear the solution of anti-DIG antibodies conjugated with alkaline phosphatase, the tube was spun down before use in the antibody binding step.

Antibody Binding

The plant samples were transferred into small glass vials containing BS for the antibody binding reaction. The previously preabsorbed anti-DIG antibody alkaline phosphatase conjugate was further diluted 1:50 to achieve a total dilution of 1:2000 and added to the plant samples. The antibody incubation was performed at 4° C o/n (19 h).

Washing

The antibody solution was removed and the samples were equilibrated in BS for 10 min followed by four 15 min wash steps in PBT.

Chromogenic Reaction for Alkaline Phosphatase Conjugate

The plant samples were washed twice for 5 min in SB with 1 mM levamisole as inhibitor of endogenous alkaline phosphatases added. The color reaction was performed in SB with 1 mM levamisole and 6.6 µl/ml NBT (50 mg/ml) as well as 3.3 µl/ml X-phosphate (50 mg/ml) added. During the reaction the samples were incubated in the dark and both short and long staining reactions were performed. The short staining reaction was stopped after 45 min by rinsing the samples twice in SSB. The long staining reaction was stopped the same way after 7 h 20 min. Both sets of samples were then put in 50% glycerol followed by a transfer into 70% glycerol.

Clearing and Microscopical Observation

The samples were put on microscope glass slides into a drop of clearing agent (see 3.2.11.1) and incubated at room temperature over night. These mounted seedlings were examined under the microscope and photographed with a Nikon SMS-10A and a Nikon EFD-3.

3.2.13. PCR-based Screening

A Mutagenesis System for *Arabidopsis* at the Max-Planck-Institut (MPI) für Züchtungsforschung using *En-1* Elements (AMAZE) in Köln, Germany was used with friendly permission of Prof. Dr. Klaus Palme The resources of AMAZE included a collection of *Arabidopsis* seed stocks and genomic DNA preparations from about 3000 lines carrying approximately 15,000 insertions of the maize transposable elements *En-1*. Within these lines the insertion of the *En-1* elements into the *Arabidopsis* PP2A genes was investigated by PCR screens using primers complimentary to the edges of the *En-1* element and primers complimentary to all five *A. thaliana* PP2A catalytic subunits. PCR conditions used during screening were as follows: 50 - 80 ng of template DNA were added to 20 pmol each of forward and reverse primer, 50 µM dNTPs and 1.25 units of Boehringer Taq Gold polymerase in 1 x reaction buffer (Roche Boehringer Mannheim) containing 1.5 mM $MgCl_2$. The reactions were heated at 85° C for 1 min, and in 40 cycles the samples were denatured at 94° C for 40 sec, annealed at 60° or 65° C for 1 min and elongation was performed at 72° C for 2 min. The reaction was concluded with a final extension step at 65° C for 5 min.

3.2.14. Filter-Lift Assay for β-Galactosidase Activity

X-gal solution:	25 mg/ml	
Z Buffer:	60 mM	Na_2HPO_4
	40 mM	NaH_2PO_4
	10 mM	KCl
	1 mM	$MgSO_4$
Added fresh:	50 mM	β-mercaptoethanol

Yeast colonies were grown on plates containing appropriate selection media. A nitrocellulose filter was put onto the plate and yeast cells were lifted off by removing the filter. The filter was put face up on a sheet of aluminum foil with bend up edges and placed into liquid nitrogen to freeze the filter. Whatman filters were put into a petri dish and soaked with Z buffer with freshly added β-mercaptoethanol and 350 µg/ml X-gal solution. The aluminum "boat" with the filter was removed and thawed at room temperature. The filter was then face down put onto a Whatman filter presoaked in buffer Z with β-mercaptoethanol and X-gal. The sandwich of nitrocellulose and Whatman filter was wrapped into Saran Wrap and incubated at 30° C for 1-4 h until blue color developed.
Cells showing blue coloring were then scraped of the nitrocellulose and patched onto plates with appropriate selection media.

4. Results and Discussion

4.1. Studies to Analyze the Regulation of the *Arabidopsis thaliana HEMA1* Gene Promoter

In chloroplasts, the universal precursor of tetrapyrroles, 5-aminolevulinic acid (ALA), is derived from the glutamate of Glu-tRNA^{Glu} via the two-step C_5-pathway (Beale and Castelfranco, 1974). Glutamyl-tRNA reductase (GluTR), which catalyzes this first step in the C_5-pathway, was shown to be the target of several regulatory mechanisms (Cornah *et al*., 2003). In *A. thaliana*, GluTR is encoded by a total of three genes called *HEMA1, HEMA2* and *HEMA3*. *HEMA3* was not known at the time of this study and has so far only been identified through database searches of the *Arabidopsis* genome (McCormac and Terry, 2002). The expression of *HEMA2* had been shown to occur at low levels in roots and flowers (Kumar *et al.*, 1996a). In contrast, *HEMA1* responded to light and had a distinct pattern of gene expression in all tissues, but was most abundant in leafs (Ilag *et al.*, 1994).

The initial goal of this study was to analyze the *HEMA1* promoter. By using promoter deletions fused to the β-glucuronidase (GUS) reporter gene system, it was attempted to identify DNA-elements important for reporter gene expression and consequently for gene expression and light regulation of *HEMA1*.

4.1.1. Light Regulation of *HEMA1* Gene Transcription

Previous *HEMA1* expression studies had been preformed through Northern blot analysis (Ilag *et al.*, 1994; Kumar *et al.*, 1996a) and showed *HEMA1* mRNA expressed in all tissues examined (root, stem, leaf and flower), with the highest level of transcript detected in leafs. *HEMA1* transcripts were detected from *Arabidopsis* plants grown in white light, whereas transcripts were not detectable after light-grown plants were transfered into the dark.

Before studying the expression pattern of *HEMA1* promoter deletion constructs through the use of reporter gene GUS, the localization of *HEMA1* mRNA expression in *Arabidopsis* wild-type seedlings was examined. For this purpose, whole-mount *in situ* hybridization was performed. The method employed is based on experiments performed by de Almeida Engler *et al.*, 1994 and Cox *et al.*, 1984. This technique allows for detection of specific mRNAs in morphologically preserved tissue like whole seedlings. For this purpose, digoxigenin (DIG)-labeled antisense and sense *in vitro* transcripts of *HEMA1* cDNA were created and used as probes to detect *HEMA1* mRNA.

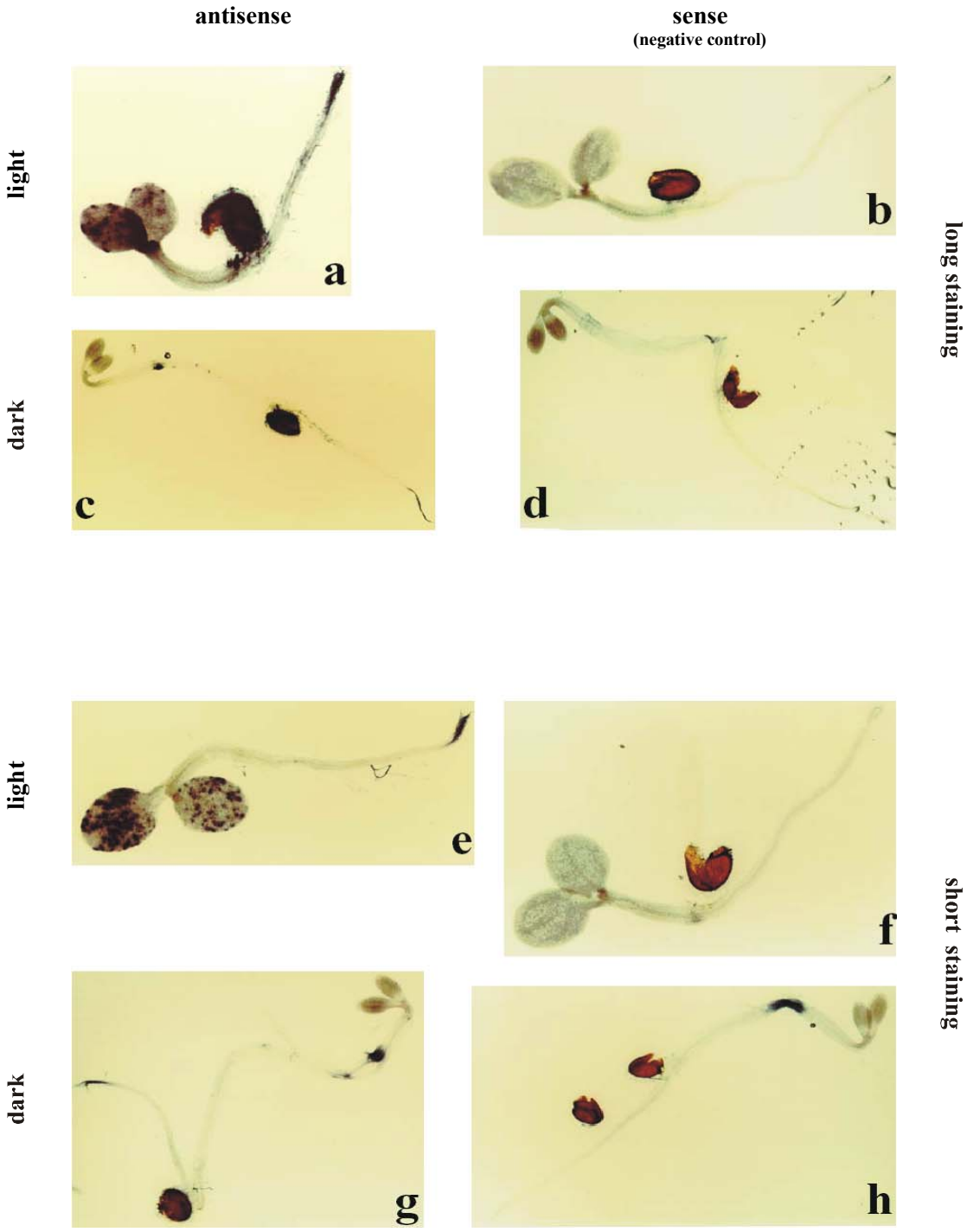


Figure 7: Light-dependent localization of *HEMA1* mRNA in *Arabidopsis* seedlings

Whole mount *in situ* hybridization was performed on five days old *Arabidopsis* seedlings. Both sense and antisense transcripts were produced from *HEMA1* cDNA, DIG-labeled and hybridized to *A. thaliana* tissue *in situ*.

Seedlings were grown in light (panels a, b and e, f) or in the dark (panels c, d and f, g) and either incubated with DIG-labeled antisense (panels a, c and e, g) or sense transcript (panels b, d and f, h). The staining reactions were performed for 45 min (panels a - d) or for 7.5 h (panels e - f).

The sense transcript served as negative control to visualize unspecific background staining. Specific staining for *HEMA1* was observed in leafs and root tips in light grown seedlings (panels a and e) and also in the root tip of dark grown seedlings (panels c and g).

The antisense transcript is able to specifically hybridize to the *HEMA1* mRNA, whereas the sense transcript serves as negative control to detect background staining. By using this technique, the localization of *HEMA1* gene expression within the intact seedling was determined. Furthermore, the pattern of *HEMA1* gene expression in light grown plants was compared to seedlings grown in the dark.

Arabidopsis seedlings were grown for five days on MS media plates either in the light or in the dark. The described protocol for *in situ* hybridization was followed and the seedlings were prepared for efficient probe penetration by a Proteinase K digestion step during pre-hybridization treatment. The DIG-labeled RNA probes were added during hybridization with half the samples being hybridized to the antisense probe and half to the sense probe. The DIG signal was detected through anti-DIG antibody conjugate binding. The bound antibody was visualized by a chromogenic reaction of the alkaline phosphatase conjugate with the staining reaction being performed for 45 min (short staining) or 7 1/2 h (long staining). To detect the specific staining pattern, mounted and cleared samples were examined under the microscope as whole seedlings (see Fig. 7).

Overall, there seemed to be little difference between the two time periods of staining. Examination of whole seedlings revealed expression of the *HEMA1* transcript in the cotyledons in light-grown seedlings, whereas dark-grown seedlings did not show staining in cotyledons above the level of background staining observed with the sense probe (see Fig. 7). In contrast, both light-grown and dark-grown seedlings showed strong staining at the root tip.

The observed localization patterns of *HEMA1* transcript matched a photosynthetic cell type-specific expression with staining signals being strong in mesophyll cells and guard cells which contain photosynthetically active chloroplasts. The observed staining in light-grown seedlings compared to the lack of staining in dark-grown seedlings further confirmed the already described light-regulation of *HEMA1* gene expression determined by Northern blot analysis (Ilag *et al.* 1994, Kumar *et al.*, 1996a). However, in contrast to the Northern blot results where mRNA was detected in all tissues including stems, no accumulation of transcript above background staining was detected in the hypocotyls. This could be due to different sensitivity levels of the two techniques or due to different developmental stages of the plants examined as the seedlings used for *in situ* hybridization were five days old whereas the plants used for RNA isolation were five weeks old (Ilag *et al.*, 1994).

The observed staining at the root tip appeared to be light-independent and in a region of strong cell division. A high rate of cell division is accompanied by increased heme utilization and production which might explain the observed *HEMA1* induction at the root tip. This second area

of staining gave an indication of two separate expression patterns of *HEMA1*. One basal, light-independent expression that covers the need for ALA in the dark and a light-induced *HEMA1* expression that enables the plant to respond to higher demand of ALA for the production of chlorophyll in light.

4.1.2. Identification of Potential Regulatory Elements in the *HEMA1* Gene Promoter Sequence

With the goal of this study being the identification and localization of promoter elements, the upstream sequence of the *HEMA1* gene was examined for elements necessary and able to regulate the level of expression. Since the exact length of the *HEMA1* promoter is not known, approximately 3 kb of upstream sequence was used to create deletion constructs.

HEMA1 promoter deletion:*gusA* fusions were constructed by Dr. A.M. Kumar and kindly donated for these studies. The deletions were created by endonuclease digest and selected by approximate size on an agarose gel. Deletions D1-D6 had a size difference of about 0.5 kb and were subcloned into the Bluescript vector pBSK$^-$. Through an engineered restriction site for *Nco*I the bacterial reporter gene β-glucuronidase (GUS) was fused to the promoter sequence deletions, thus replacing the protein coding sequence of *HEMA1* by the *gusA* coding sequence.

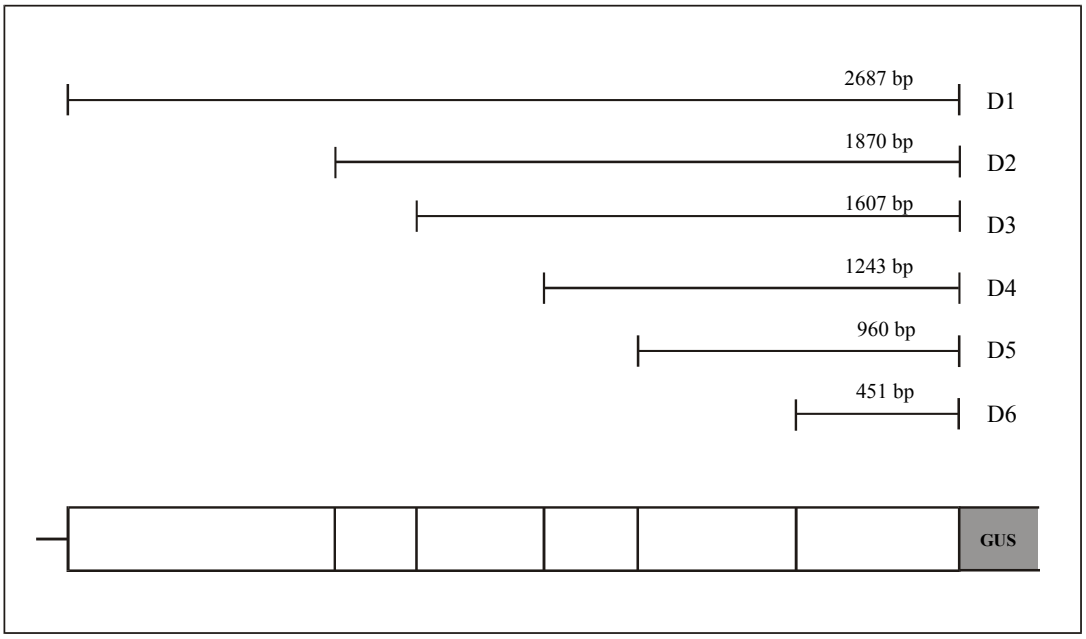


Figure 8: *HEMA1* promoter deletions fused to the GUS reporter gene
Graphical depiction of upstream sequence deletions used for promoter deletion studies. The lengths of the *HEMA1* upstream regions calculated from the translational start codon are given for the reporter gene fusions D1 - D6.

As *A. thaliana* is lacking intrinsic GUS activity and plants expressing GUS are normal, healthy and fertile, the *E. coli* β-glucuronidase gene can be used as gene fusion marker to measure gene expression in *Arabidopsis* (Abel and Theologis, 1994; Jefferson *et al.*, 1987).

As part of this study, the entire upstream sequence for each construct was determined by sequence analysis and the exact sizes of the deletion constructs are shown in Fig. 8. The longest construct D1 had a total of 2697 bp of upstream sequence. D2 contained 1870 bp of upstream sequence, D3 1607 bp, D4 1243 bp, D5 960 bp and D6 contained the shortest upstream sequence with only 451 bp. The entire upstream sequence was submitted to GenBank and designated the Accession No. AF295364.

Since the completion of the practical part of this work, the entire genomic sequence of *A. thaliana* has been published (The Arabidopsis Genome Initiative, 2000). The analysis of the *Arabidopsis* genome revealed that the *HEMA1* gene is located on chromosome 1 and has been assigned the MIPS code At1g58290 (see Fig. 9). The gene is located between a gene encoding a protein of unknown function and a gene for a putative heme oxygenase. The most distal part of the *HEMA1* upstream sequence used in this analysis is part of the putative heme oxygenase gene. An overlap between regulatory sequence and gene sequence is not unexpected, considering the relatively small size of the *Arabidopsis* genome and the expected number of genes encoded within (The Arabidopsis Genome Initiative, 2000).

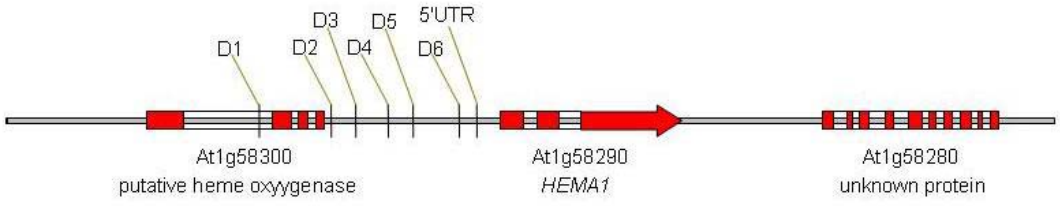


Figure 9: Genetic location of the *HEMA1* gene
Partial map of *A. thaliana* chromosome 1 showing the *HEMA1* locus encoding glutamyl-tRNA reductase and the surrounding up- and downstream areas.

The entire upstream sequence showing the location of the *HEMA1* promoter deletion constructs is depicted in Fig. 11. A more detailed schematic of the shortest deletion construct D6 is shown in Fig. 10.

A sequence comparison of the *HEMA1* upstream sequence with the upstream sequences of the *HEMA2* and *GSA1* genes, the latter encoding the second enzyme of the C_5-pathway GSA-AM, displayed two areas of DNA sequence homology. As indicated in Fig. 10 by a purple box, an area of 21 bases was found within the sequence of the *HEMA1* upstream region that showed an identity

of 20 out of 21 bases to a DNA sequence found upstream of *HEMA2* (see purple stars in Fig. 10). Also, within a blue box in Fig. 10, another area of 24 bases within the *HEMA1* upstream region showed a 71% identity (17 out of 24 bases) between *HEMA1* and *GSA1* indicated by blue stars (Ilag *et al.*, 1994). These observations could indicate that both *HEMA1* and *HEMA2* as well as *HEMA1* and *GSA1* share a common element of regulation.

The transcription start site of *HEMA1* was determined by a 5' RACE approach as described in McCormac *et al.*, 2001. One major start site which conformed to the consensus motif for eukaryotic transcription start sites was found 252 nucleotides upstream of the translation start site. Also, two additional minor start sites were found as depicted in Fig. 10. Analysis of the sequence data revealed several candidates for TATA boxes, however none was localized in the expected position between -50 bp to -30 bp in relation to the experimentally determined transcription start sites. As described in the literature (Zhu *et al.*, 1995), TATA boxes cooperate with an initiator element to achieve a correct placement of the initiation site and direction of transcription complex assembly. Initiator elements overlap the transcription start site and have a consensus sequence of CA at -1 and +1. There are different classes of initiator elements and one class of strong elements

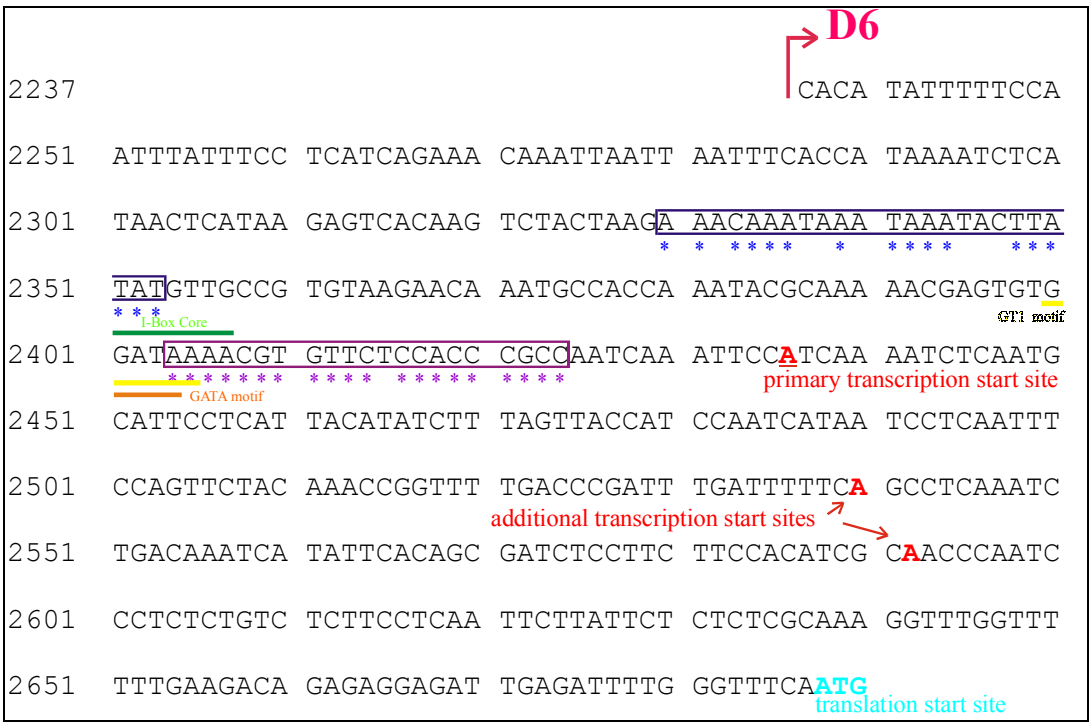


Figure 10: Promoter deletion construct D6 indicating the transcriptional start sites of *HEMA1*
Detailed schematic representation of the shortest promoter deletion construct D6 showing transcription start sites (McCormac *et al.*, 2001) and potentially important regulatory *cis*-elements. A section of homology between the *GSA1* promoter is shown by a blue box with sequence identity indicated by blue stars (*) (Ilag *et al.*, 1994). A purple box shows a section of homology between the *HEMA1* and *HEMA2* promoter with purple stars (*) indicating sequence identity.

Figure 11: Complete analyzed upstream sequence of *A. thaliana HEMA1*

Nucleotide sequence of the analyzed *HEMA1* gene promoter (GenBank accession number AF295364). D1 to D6 denote the upstream end of the promoter deletions relative to the translational start codon. DNA sequences with a high degree of identity to elements with known function in light regulation (LRE - Light Responsive Elements) are indicated by colored bars below or above the sequence: — I-Box Core (consensus GATAA), — GT1 motif (consensus: GRWAAW), — GATA motif (consensus: GATA)

is able to initiate transcription in TATA less promoters. This is consistent with the occurrence of multiple start sites resulting in different length transcripts. Therefore, *HEMA1* transcription seems to be initiated by a TATA less promoter.

To further determine potential regulatory elements in the *HEMA1* promoter, the upstream sequence was compared to the PLACE database which compiles motifs found in plant *cis*-acting regulatory DNA elements (Higo *et al.*, 1998). This comparison revealed several motifs possibly involved in light regulation called Light Responsive Elements (LREs) (Terzaghi and Cashmore, 1995). These elements were distributed over all the different deletion constructs with the exception of D3. It is expected from the literature that these elements work together to confer light responsiveness (Puente *et al.*, 1996; Chattopadhyay *et al*., 1998). One of these elements, GT-1, has been described as necessary, but not sufficient for the light -response of a promoter, but has also been shown to act as silencing element depending on its cooperative interaction with other elements (Lawton *et al*., 1991; Zhou, 1999). Analysis of the upstream area also revealed the sequence to be AT rich which has been described for light-regulated promoters. AT-rich sequences seem to cooperate with other regulatory elements to confer either positive or negative regulatory effects on the expression depending on the nature of the interacting element(s) (Terzaghi and Cashmore, 1995).

These findings raised the expectation that the generated *HEMA1* promoter deletion constructs could be useful to examine differences in light response conferred by various elements contained within the *HEMA1* promoter. Therefore, the *HEMA1* promoter deletion:*gusA* constructs D1-D6 were used to study the reporter gene expression in both transient and stable transformation experiments in *A. thaliana*.

4.1.3. Transient Expression of *HEMA1* Promoter:*gusA* Fusions in *A. thaliana* Protoplasts

The isolation and transformation of mesophyll protoplasts from *A. thaliana* leaf tissue has been established as an effective tool to examine the transient expression of genes of interest. It provides a convenient alternative to stable transformation analysis (i.e. transgenic plants) and allows for a comparatively rapid assay system (Abel and Theologis, 1994; Jefferson *et al.*, 1987).

In order to analyze the different *HEMA1* promoter deletion constructs, protoplasts from seven weeks old *Arabidopsis* plants were isolated, transformed, and incubated in the light for 24 h. GUS activity was determined as rate of enzymatic 4-methyl-umbelliferone (4-MU) production per time and normalized to the total protein content of the protoplasts.

Table 1: GUS activity of cell free extracts prepared from *Arabidopsis* protoplasts after transient transformation with various *HEMA1* promoter deletion constructs

Sample	**Average GUS activity** [1] (nmol 4-MU min^{-1} mg total $protein^{-1}$)	
D1	46.0	+/- 2.9
D2	3.9	+/- 0.5
D3	26.8	+/- 1.1
D4	26.5	+/- 4.2
D5	5.5	+/- 0.7
D6	2.5	+/- 0.4
negative control [2]	0.03	+/- 0.0009
positive control [3]	709.5	+/- 24.8

[1] GUS activity was determined as rate of enzymatic 4-methyl-umbelliferone (4-MU) production per time and normalized to the total protein content of *A. thaliana* protoplasts after transient transformation. Measurements were taken in triplicates and averages and the standard error among measurements were calculated.
[2] Protoplasts only transformed with carrier DNA served to determine the background of the reaction.
[3] Protoplasts transformed with the GUS gene under the control of constitutively expressing CaMV 35S promoter served as positive control.

a. **b.**

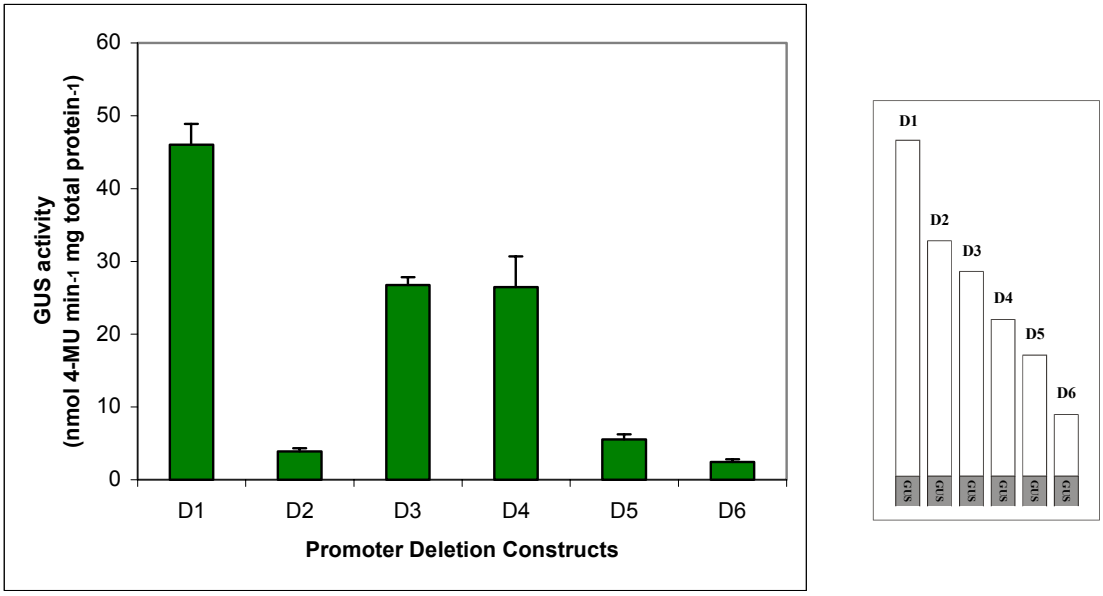


Figure 12: Transient expression of *HEMA1* promoter deletion:*gusA* constructs in leaf protoplasts in relationship to the promoter length of each construct
a. Graphical depiction of results shown in Tab.1. Protoplasts from seven weeks old *Arabidopsis* plants were isolated and transiently transformed with promoter deletion constructs D1-D6. GUS activity was measured as enzymatic 4-methyl-umbelliferone (4-MU) production per time and normalized to the total protein content of *A. thaliana* protoplasts after transient transformation. b. Schematic representation of the corresponding *HEMA1* promoter deletion:*gusA* construct.

All measurements of GUS activity in cell free extracts prepared from protoplasts carrying the various promoter deletion constructs fused to the gene for β-glucuronidase and appropriate controls were taken in triplicates. Samples were taken at three time points after GUS substrate addition (30 min, 60 min and 120 min) for each triplicate. To capture the enzymatic reaction at its linear phase, the average GUS enzyme activity for each promoter deletion construct was determined as rate of 4-MU production per minute between the 60 min and 120 min timepoints and normalized to the total protein content of the protoplast extract (see Tab. 1 and Fig. 12).

The plasmid pCG containing the GUS reporter gene under the control of the Cauliflower mosaic virus (CaMV) 35S promoter was used as a positive control. The CaMV 35S promoter is known to show constitutive, high level of expression. The negative control to determine the background activity for the reaction was performed with protoplasts carrying no plasmid DNA. Compared to the positive control (average GUS activity of 709.5 nmol 4-MU min^{-1} mg total $protein^{-1}$), the enzyme production achieved by all *HEMA1* promoter constructs was considerably lower (average enzyme activity ranged between 2.5 and 46.0 nmol 4-MU min^{-1} mg total $protein^{-1}$). However, the activities were distinctly above background (average activity of 0.03 nmol 4-MU mg total $protein^{-1}$ min^{-1}).

As can be seen in Table 1 and Fig. 12, the GUS production varied significantly for the different promoter deletions. The full-length upstream sequence represented by the promoter construct D1 showed the highest level of reporter gene expression. Interestingly, upon removal of 817 bp of upstream sequence the reporter gene expression seen with construct D2 dropped dramatically to only 8% of the level of D1 expression. However, the expression level recovered with constructs D3 and D4 which showed approximately 58% of the reporter gene expression of D1. Finally, the expression level dropped with constructs D5 and D6 to 12% and 5%, respectively, of the level seen with D1.

These results can be explained by the presence of potential enhancer and repressor elements. Responsible for the drop of expression seen with D2 could be a negative regulatory element located between the position of -1870 and -1607 bp, which itself gets suppressed by some element in the full length upstream sequence. Following the removal of this negative regulatory element in D3 and D4, an elevation of reporter gene expression occurred. Positive regulatory elements are responsible for the achieved level of reporter gene expression. These positive regulatory elements could have an additive effect which upon removal of several of these elements was responsible for the drop in expression in D5 and D6.

However, it should be noted that even with the shortest deletion construct D6, reporter gene expression above background could be achieved. The observed reporter gene expression under the control of the shortest construct D6 indicated that this 451 bp fragment contains all necessary elements for gene expression and can function as minimal promoter.

4.1.4. Expression of *HEMA1* Promoter:*gusA* in Transgenic *A. thaliana* Plants

In the previously described transient expression experiment, the protoplasts were derived from mesophyll cells isolated from *Arabidopsis* leafs. To gain further information not limited to only one tissue type, stable transformation of the *HEMA1* promoter deletion:*gusA* constructs was performed. *In vivo* analysis of transgenic plants using the previously described promoter deletions was then used to further investigate the promoter of *HEMA1*.

4.1.4.1. Generation of Stable Transgenic Lines carrying *HEMA1* Promoter:*gusA* Fusions

The *HEMA1* promoter deletion:*gusA* constructs described in 4.1.2. were subcloned into the binary vector pCIT20, amplified in *E. coli* DH5α cells and then transformed into the *Agrobacterium* strain GV3101. Vacuum infiltration was used to introduce *A. tumefacies* cells carrying the binary vector with the *HEMA1* promoter deletion constructs into *Arabidopsis* plants. The strain GV3101 contains the virulence gene Ti on the helper plasmid pMP90. Therefore, a transfer of the T-DNA cassette that included a gene conferring hygromycin resistance from pCIT20 into the genome of *A. thaliana* plants was possible.

Table 2: Number of isolated transgenic *A. thaliana* lines after vacuum transformation with different GUS reporter gene constructs.

***HEMA1* Promoter Deletion Construct**[1]	**Number of Transgenic Lines**
D1	25
D2	16
D3	5
D4	24
D5	10
D6	19

[1] For detailed description of the *HEMA1* promoter deletion constructs consult 4.1.2. and Fig. 8.

Potential transgenic lines were selected on plates containing hygromycin and the plantlets showing resistance were transfered into soil. The plants were then grown under normal conditions and the seeds of the F1 generation were harvested.

Approximately 9,000 to 10,000 seeds per promoter deletion construct were tested in this manner resulting in 2-5 transgenic lines for only 3 of the 6 promoter deletions constructs. Due to this insufficient number of transgenic lines after first the round of transformation, a second vacuum infiltration following the same protocols was performed. Both rounds of vacuum infiltration resulted in a total of 99 transgenic lines with 5-25 lines per construct (see Tab. 2).

4.1.4.2. Analysis of Transgenic Plants carrying *HEMA1* Promoter:*gusA* Fusions

Independent homozygous lines carrying the *HEMA1* promoter deletion constructs were obtained for further analysis. Only thereby, it can be ensured that no variation of gene dosage between heterozygote and homozygote expression interferes with the measurements of GUS reporter gene expression.

4.1.4.2.1. Segregation Analysis to Identify Homozygote Lines

In order to ensure integration of the T-DNA into the *A. thaliana* genome and proper genetic transfer into the next generation, a segregation analysis was performed. To test segregation, 50 seeds of the F1 generation were put onto selection plates. Seedlings that showed resistance against hygromycin were counted, since the antibiotic resistance indicated that the T-DNA, which also included the *HEMA1* promoter deletion constructs, had successfully integrated into the genomes of the *Arabidopsis* plants.

Table 3: Summary of segregation analysis and total number of independent homozygous lines

promoter deletion construct [1]	number of trans-genic lines (F1)	number of lines with 3:1 segregation (F2)	number of independent homozygote lines [2] (F3)
D1	25	8	3
D2	16	3	3
D3	5	3	3
D4	24	12	3
D5	10	4	4
D6	19	7	3

[1] For detailed description of the *HEMA1* promoter deletion constructs consult 4.1.2. and Fig. 8.
[2] Independence of lines was determined by Southern blot analysis (see 4.1.4.2.2.).

Lines of the F2 generation that showed a 3:1 segregation of the antibiotic resistance were followed into the F3 generation. Tab. 3 shows a summary of the segregation analysis for each construct. All lines selected from the F2 generation that showed 75% segregation are listed. F3 lines showing hygromycin resistance in all seedlings were then counted as homozygous lines. This does not imply that these lines were independent nor does it rule out multiple T-DNA insertions. However, as described in McCormac *et al*., 2001, all homozygous lines used for further GUS expression experiments were crossed with *Arabidopsis* wild-type plants and showed a 3:1 segregation of antibiotic resistance indicating a single T-DNA insertion and truly homozygous lines.

4.1.4.2.2. Determination of Independent Lines

Southern blot analysis was performed to determine if the homozygous transgenic *Arabidopsis* lines obtained in the F3 generation were in fact independent. Genomic DNA from leaves of transgenic plants in the F3 generation was isolated. *Hind*III was selected as restriction enzyme since the *HEMA1* promoter deletion:*gusA* constructs did not possess a cutting site for this enzyme, but at the same time the restriction enzyme has sufficient targets within the genomic DNA of *A. thaliana* Columbia ecotype. A Southern blot was performed using the smallest *HEMA1* promoter deletion fragment D6 as probe. Different sized fragments were used as indication for independent insertions of the *HEMA1* promoter deletion:*gusA* constructs into the *Arabidopsis* genome following vacuum transformation (data not shown).

As shown in the far left column of Tab. 3, at least three independent homozygous transgenic *Arabidopsis* lines for each *HEMA1* promoter deletion construct were obtained.

All lines used for further experiments produced healthy plants showing normal growth, thereby indicating that no essential genes were disrupted by the insertion of the T-DNA.

4.1.5. Localization of *HEMA1* Promoter Driven Expression in Transgenic Plants

Independent, homozygous transgenic *Arabidopsis* lines expressing the reporter gene β-glucuronidase (GUS) driven by different *HEMA1* promoter deletions were used to identify and localize important promoter elements. The expression of the reporter gene GUS can be visualized by using X-Glucuronide as substrate for the enzyme resulting in blue staining. By looking at whole seedlings stained in this manner, the patterns resulting from different *HEMA1* promoter deletion constructs were compared for possible differences in localization and, to a certain degree, level of staining.

To examine the localization of reporter gene expression, seeds from independent transgenic lines were plated onto MS plates containing 1% agar, vernalized in the dark at 4° C for three days and grown in light or dark for four days. The seedlings were then carefully removed from the plates and stained. Different timepoints of staining were taken by removing the seedlings out of the staining solution and placing them onto a microscope slide containing a drop of clearing agent. A comparison of light-grown to dark-grown seedlings carrying the different *HEMA1* promoter deletion:*gusA* constructs was performed at timepoints of 1 h, 4 h, 8 h and 24 h of staining.

Representative samples of plants carrying the six different *HEMA1* promoter deletion constructs are shown in Fig. 13. Panels a - c show light-grown seedlings carrying the longest *HEMA1* promoter deletion D1 at different timepoints of staining. Staining was weakest at 1h of staining (panel a) and became stronger at 4 and 8 h (panels b and c, respectively). This pattern was also shown in an identical way by seedlings from different transgenic lines and seedlings carrying other promoter deletion constructs (data not shown). Independent of the promoter deletion construct carried by the seedling, the strongest development of blue color was seen at the 24 h timepoint of staining (panels D1 - D6).

A direct comparison of light-grown seedlings carrying the different *HEMA1* promoter deletions constructs D1 - D6 is shown in panels D1 to D6. All seedlings were stained for 24 h and showed very little difference in the overall pattern of GUS expression. The pattern showed staining throughout the entire seedling with very strong expression in the cotyledons including the vascular tissue. The staining appeared weaker throughout the upper part of the hypocotyl and increased dramatically at the hypocotyl root junction and at the site of lateral root primordia emergence. The entire root showed GUS expression, however no particularly strong staining could be noted at the root tip. No deviations from this overall pattern were observed depending on the different *HEMA1* promoter deletion constructs. However, the intensity of staining appeared to differ between constructs indicating quantitative differences in the level of reporter gene expression. The level of GUS expression appeared strongest with the longest *HEMA1* promoter deletion construct D1, weaker in D2, again stronger in D3 and D4 and was then getting weaker in D5 and D6. These differences in intensity reflected the results of the transient expression of the *HEMA1* promoter deletion:*gusA* constructs in protoplasts (see 4.1.3.). However, to conclusively show these quantitative differences in reporter gene expression driven by the different *HEMA1* promoter deletion:*gusA* constructs, fluorometric GUS assays were performed (see 4.1.6., 4.1.7. and 4.1.8.).

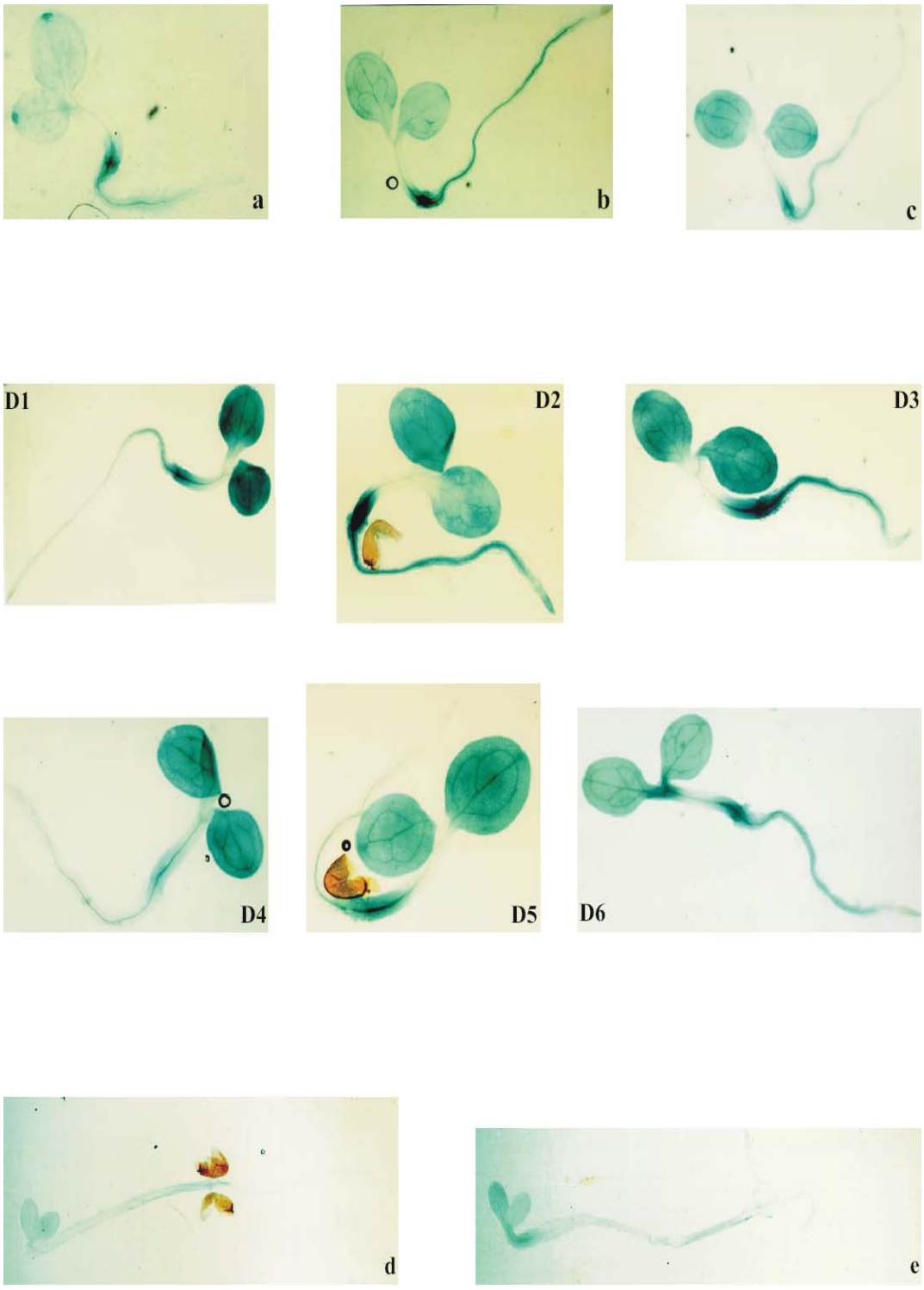


Figure 13: Histochemical GUS analysis of transgenic *A. thaliana* seedlings

Four days old transgenic *Arabidopsis* seedlings carrying *HEMA1* promoter deletion:*gusA* constructs were stained using X-Glucuronide as substrate.

Panels a - c show light-grown seedlings carrying the longest *HEMA1* promoter deletion:*gusA* construct D1 at staining timepoints of 1h, 4 h and 8 h, respectively. Panels D1 - D6 show light-grown seedlings carrying the *HEMA1* promoter deletion:*gusA* constructs D1-D6, respectively, stained for 24 h. Panels d and e show dark-grown seedlings carrying the longest *HEMA1* promoter deletion:*gusA* construct D1 at staining timepoints of 8 and 24 h, respectively.

Dark-grown seedlings carrying the longest *HEMA1* promoter deletion D1 stained for 8 h and 24 h are shown in panels d and e, respectively. These seedlings showed a weak, but even distribution of blue color throughout the entire seedling without showing strong staining at the root tip. The staining was considerably weaker than the staining obtained in light-grown seedlings at comparable timepoints (panels c and D1).

Overall, the staining pattern was similar to the pattern observed following *in situ* hybridization (see 4.1.1.). There, the accumulation of native *HEMA1* transcript was visualized in a similar pattern with the exception of the light-independent strong staining of the root tip. This area was clearly stained in light and dark grown seedling following *in situ* hybridization, however no blue color was detected with GUS staining of transgenic plants.

It is unclear, why *in situ* hybridization showed a light-independent localization of *HEMA1* mRNA in the root tip, while GUS staining of transgenic plants showed no indication of any reporter gene expression in the area. However, *in situ* hybridization visualizes level of transcripts whereas the histochemical GUS analysis localizes GUS protein production. Therefore, translational and post-translation regulation processes could be responsible for the observed differences in the pattern between the two used techniques.

4.1.6. Light Response of *HEMA1* Promoter Deletions

HEMA1 gene expression shows a light response, as previously described by Ilag *et al.*, 1994 and Kumar *et al.*, 1996a and also observed in this study (see 4.1.1. and 4.1.5.). More transcript and higher GUS reporter gene expression was observed in plants grown in the light compared to etiolated seedlings. To quantitate the observed differences in transgenic plants, independent homozygote transgenic lines were subjected to different light treatments and a fluorometric GUS assay was performed.

Approximately 100 seeds of several homozygote lines containing different *HEMA1* promoter deletion:*gusA* constructs were each plated onto duplicate plates. The plates were vernalized in the dark at 4° C for three days, given a four hour white light treatment and the seedlings were then grown for ten days either in the dark or in white light (16 h white light and 8 h dark day cycles). The complete seedlings were harvested, frozen in liquid nitrogen and cell-free protein extracts were prepared. The protein concentration of the extracts was determined and GUS activity was measured as described in Materials and Methods.

GUS activity was determined as rate of enzymatic 4-methyl-umbelliferone (4-MU) production per minute and normalized to the total protein content of each extract. Several independent homozygote transgenic lines were tested for each *HEMA1* promoter deletion construct. As a representative result, data from one transgenic line for each construct is shown in Tab. 4 and Fig. 14 shows a graphical depiction of the results.

There were clear variations among the absolute values in different transgenic lines, but the overall patterns remained the same with the exception of D5. Transgenic lines carrying *HEMA1* promoter deletion constructs D5 showed a very high variation between lines possibly due to different sites of T-DNA mediated construct insertion. Therefore, the results obtained with D5 could only be interpreted in a limited manner.

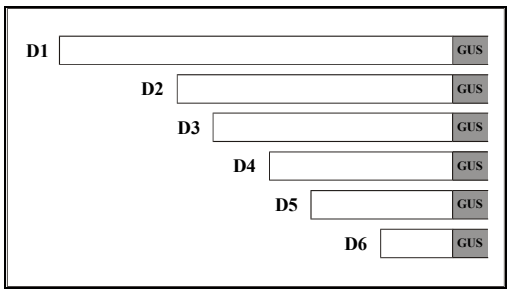
Looking at the measured GUS activities of transgenic plants grown in the light (see Tab. 4 and Fig. 14, panel b), a strikingly similar pattern emerged as seen following transient expression of *HEMA1* promoter deletion:*gusA* constructs in leaf protoplasts. The highest level of GUS activity was seen with the longest *HEMA1* promoter construct D1, followed by a drop in GUS activity with D2 to about 22% of the value of D1. As noted before following transient transformation with *HEMA1* promoter constructs, the GUS activity recovered with D3 to 46% of the D1 level. In this experiment, the GUS activity of D4 again dropped to 16% of the level of D1,

Table 4: GUS activity of cell-free extracts prepared from transgenic *Arabidopsis* plants carrying different *HEMA1* promoter deletion:*gusA* constructs.

	10 days light	**10 days dark**	
Promoter Deletion Construct	average GUS activity [1] (pmol 4-MU mg protein^{-1} min^{-1})	average GUS activity [1] (pmol 4-MU mg protein^{-1} min^{-1})	fold induction between expression in dark and in light
D1	485.2 +/- 42.5	25.1 +/- 1.2	19
D2	108.0 +/- 11.7	9.3 +/- 0.3	12
D3	221.5 +/- 1.5	15.8 +/- 1.5	14
D4	78.4 +/- 8.1	3.9 +/- 0.2	20
D5	117.1 +/- 3.4	25.3 +/- 0.9	5
D6	53.7 +/- 3.0	5.8 +/- 0.4	9

[1] GUS activity was measured as rate of enzymatic 4-methyl-umbelliferone (4-MU) production per min and normalized to the total protein content of each extract. Measurements were taken in duplicates or quadruplicates and averages and standard error (SE) of measurements of one transgenic line were calculated.

a.



b.

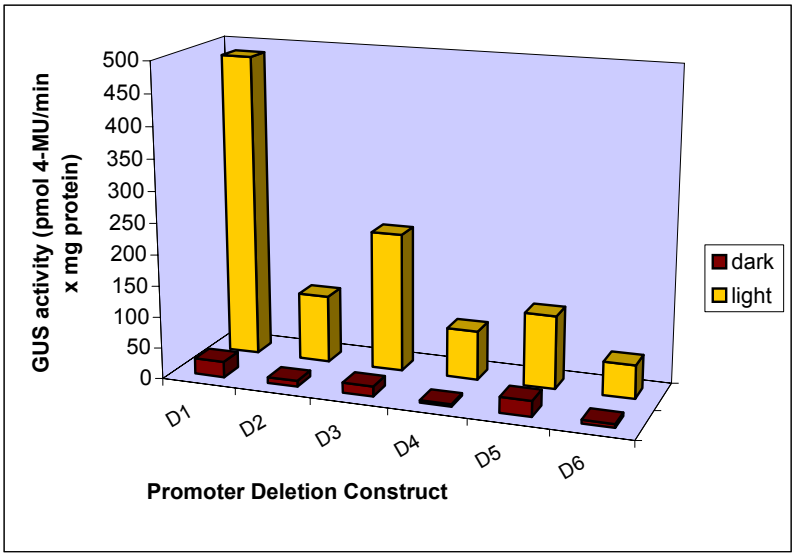


Figure 14: GUS activity of transgenic *Arabidopsis* plants

Graphical depiction of results shown in Tab. 4. Transgenic *Arabidopsis* plants carrying *HEMA1* promoter deletion:*gusA* constructs were grown in ten days light or ten days dark. Protein extracts were prepared and fluorometric GUS assays were performed. GUS activity was measured as rate of enzymatic 4-methyl-umbelliferone (4-MU) production per min and normalized to the total protein content of each extract.
a. Schematic of *HEMA1* promoter deletion:*gusA* constructs. b. Comparison of GUS activities of plants grown in ten days light to plants grown in ten days dark within the same transgenic line carrying a *HEMA1* promoter deletion construct.

whereas the GUS activity of D5 increased to 24% of D1. Again, the lowest level of GUS activity was seen with the shortest *HEMA1* promoter deletion:*gusA* construct D6 which amounted to about 11% of the activity level of D1.

GUS activities from transgenic plants grown in the dark mimicked the overall patterns seen with light grown plants, albeit at a significantly lower level of reporter gene expression. The overall values of fold induction between expression in dark-grown and light-grown transgenic plants was different for the deletion constructs and varied between 5 and 20. The strongest reaction to light could be seen with deletion constructs D1 and D4 with a 19 and 20 fold induction, respectively,

between GUS reporter gene expression in dark- and light-grown transgenic plants (see Tab. 4). The constructs D5 and D6 showed the weakest reaction to light.

When comparing the pattern of light grown transgenic plants with the pattern from the transient expression experiment, the promoter deletion constructs D4 and D5 show a slightly different behavior. In protoplasts, the level of expression for D4 stayed at the same level as D3 and D5 showed a further drop in activity. These constructs seem to have reversed activity levels in this experiment. This, however, might be due to the degree of variation seen between lines carrying the *HEMA1* promoter deletion construct D5 (data not shown).

The patterns of GUS activity obtained from light-grown transgenic plants and transient transformed protoplast matched in the highest reporter gene activity being generated by the longest *HEMA1* promoter deletion construct D1 and the lowest activity coming from the shortest construct D6. Both patterns also showed the drop of expression driven by the construct D2 and showed a recovery of the GUS activity level upon removal of the next 263 bp (construct D3). This can be seen as mounting evidence of a negative regulatory element between the positions -1870 bp and -1607 bp which causes the reporter gene expression to drop. The sequence 817 bp further upstream of this element seems to be able to counteract this down-regulatory effect resulting in the highest expression level. Also, upon further removal of the negative effect in construct D3, the expression is able to recover to a certain extend, however not to the same level achieved by the longest promoter deletion construct.

Both experiments showed clearly that the shortest promoter deletion construct D6 still contains all necessary elements to function as a minimal promoter. Reporter gene GUS expression was seen in both dark-grown and light-grown plants. Importantly, considering the nine fold induction between expression in dark-grown and light-grown plants, the minimal promoter D6 contains essential elements capable of light response.

For these described experiments, whole seedlings were used to prepare the tested protein extract. To further describe the actual location of the light response, McCormac *et al.,* 2001, measured the light-mediated effect on extracts prepared from cotyledons, hypocotyl and root of transgenic *Arabidopsis* plants carrying *HEMA1* promoter constructs. The *HEMA1* promoter driven light-response was noticeably strongest in the cotyledons. Some increase compared to expression in dark-grown plants was seen in hypocotyls, however the overall expression level was low compare to the cotyledons. No light effect could be noted on the GUS reporter gene expression in roots. This matches the observed results of the *in situ* hybridization where the localization of *HEMA1* mRNA indicated that the actual light-responsive transcription occurs in leafs. Light-independent transcript accumulation was seen in the root tip. This again confirmed that the *HEMA1* promoter

is capable of maintaining a basal level of transcription, evident by the light-independent reporter gene expression in dark-grown plants. However, a very significant increase of expression occurs in the light and further experiments were performed to describe the photoreceptor systems involved in this light response.

4.1.7. Differential Light Response of *HEMA1* Promoter Deletions

Plants monitor light through different photoreceptors which are characterized by the wavelength of light they perceive and by the different signal transduction cascades they initiate (recently reviewed by Sullivan and Deng, 2003). In order to elucidate the photoreceptors involved in the light response observed on the *A. thaliana HEMA1* promoter, homozygote transgenic lines were subjected to light of different wavelengths and subsequent fluorometric GUS assays.

About 100 seeds each of homozygote transgenic *Arabidopsis* lines containing different promoter GUS constructs were sterilized, distributed onto plain MS plates with 0.8% agar and vernalized in the dark at 4° C for two days. All plates were then moved into continuous light of different wavelengths for seven days or incubated in the dark for seven days after which the phenotypes of the seedlings were noted.

Overall, the observed phenotypes for the transgenic seedlings matched the expected effects of the light of different wavelengths (see Whitelam and Devlin, 1998). Seedlings grown in the dark showed etiolated phenotypes with elongated hypocotyls. Hooks and cotyledons were small and lacked green color. Seedlings grown in red light showed elongated hypocotyls, but normal open and green cotyledons. Far-red light created very small seedlings that showed slightly yellow but otherwise normal cotyledon. Seedlings grown in blue light showed slightly elongated hypocotyls and should have shown yellow cotyledons. This, however, was the only exception from the expected phenotypes as the seedling in this experiment had green cotyledons with a normal appearance.

The seedlings were harvested as whole plants, frozen in liquid nitrogen and protein extracts were prepared. The protein concentration of the extracts was determined and GUS activity was measured as described in Materials and Methods. GUS activity was determined as rate of enzymatic 4-methyl-umbelliferone (4-MU) production per minute and normalized to the total protein content of each extract. As before, several independent homozygote transgenic *Arabidopsis* lines were tested and as representative results, data from one typical transgenic line for each construct is shown in Tab. 5. Fig. 15 shows a graphical depiction of the results.

As seen before, all dark-grown transgenic plants carrying *HEMA1* promoter deletion constructs showed a low level of reporter gene GUS expression. The same transgenic lines showed a significant increase in GUS activity when grown in seven days continuous white light. Compared to the respective value observed in dark-grown plants, all transgenic plants also showed an increase in reporter gene expression when grown in seven days continuous red light, far-red light or blue light. The levels of GUS activities driven by the different promoter deletions varied in absolute values, however the overall patterns obtained in different light qualities were very similar. The relative responsiveness to red light, far-red light and blue light compared to white light and expression in dark-grown plants appeared to be nearly the same with all *HEMA1* promoter deletion constructs.

The strongest response to red light was seen with constructs D1, D2 and D6 where the level of GUS reporter gene expression was even slightly higher than in white light. All others showed a reporter gene activity that was about the same as the GUS activity in white light. All constructs showed an elevated level of GUS activity in extracts prepared from plants grown in continuous far-red light compared to dark-grown plants. However, this activity was only between 13 - 6% of the observed GUS activity of the same transgenic line grown in red light. All transgenic plants containing *HEMA1* promoter deletion constructs showed a response to blue light which with the exception of D3 was considerably higher than the response to far-red light.

McCormac *et al.,* 2001, also examined the light response of the *HEMA1* promoter by Northern blot analysis. The detected level of *HEMA1* mRNA was low in dark-grown seedlings and showed a dramatic increase when plants were exposed to irradiation by white light, red light, far-red light and blue light. This confirms the results of this study in fluorometric GUS assays with transgenic plants carrying *HEMA1* promoter constructs.

The observed responses to red light and far-red light implicate the phytochrome system in the light response of the *HEMA1* promoter, whereas the response to blue light also indicates a role for blue light receptors such as cryptochromes and phototropins.

Table 5: GUS activity of transgenic *Arabidopsis* plants carrying *HEMA1* promoter deletion:GUS constructs exposed to continuous light of different wavelengths.

Light Conditions	Promoter Deletion Construct	D1	D2	D3	D4	D5	D6
7 days dark	GUS activity [1] (pmol min^{-1} mg^{-1})	29.5	9.1	12.9	8.8	1.8	2.6
	SE	+/- 1.2	+/- 0.1	+/- 0.1	+/- 0.1	+/- 0.1	+/- 0.3
7 days white light	GUS activity (pmol min^{-1} mg^{-1})	412.9	265.0	492.7	432.5	151.4	59.0
	SE	+/- 32.4	+/- 7.3	+/- 83.5	+/- 65.9	+/- 40.6	+/- 7.8
7 days red light	GUS activity (pmol min^{-1} mg^{-1})	521.3	418.0	462.7	561.9	131.8	112.6
	SE	+/- 7.9	+/- 20.0	+/- 11.9	+/- 44.9	+/- 4.1	+/- 5.5
7 days far-red light	GUS activity (pmol min^{-1} mg^{-1})	128.9	108.3	167.3	92.8	17.4	24.0
	SE	+/- 8.9	+/- 7.5	+/- 2.5	+/- 5.9	+/- 3.0	+/- 1.8
7 days blue light	GUS activity (pmol min^{-1} mg^{-1})	291.9	234.6	188.1	382.0	81.3	66.8
	SE	+/- 48.1	+/- 4.0	+/- 8.6	+/- 21.2	+/- 10.7	+/- 1.3

[1] GUS activity was measured as rate of enzymatic 4-methyl-umbelliferone (4-MU) production per min and normalized to the total protein content of each extract. Measurements were taken in duplicates or quadruplicates and averages and their standard error (SE) were calculated.

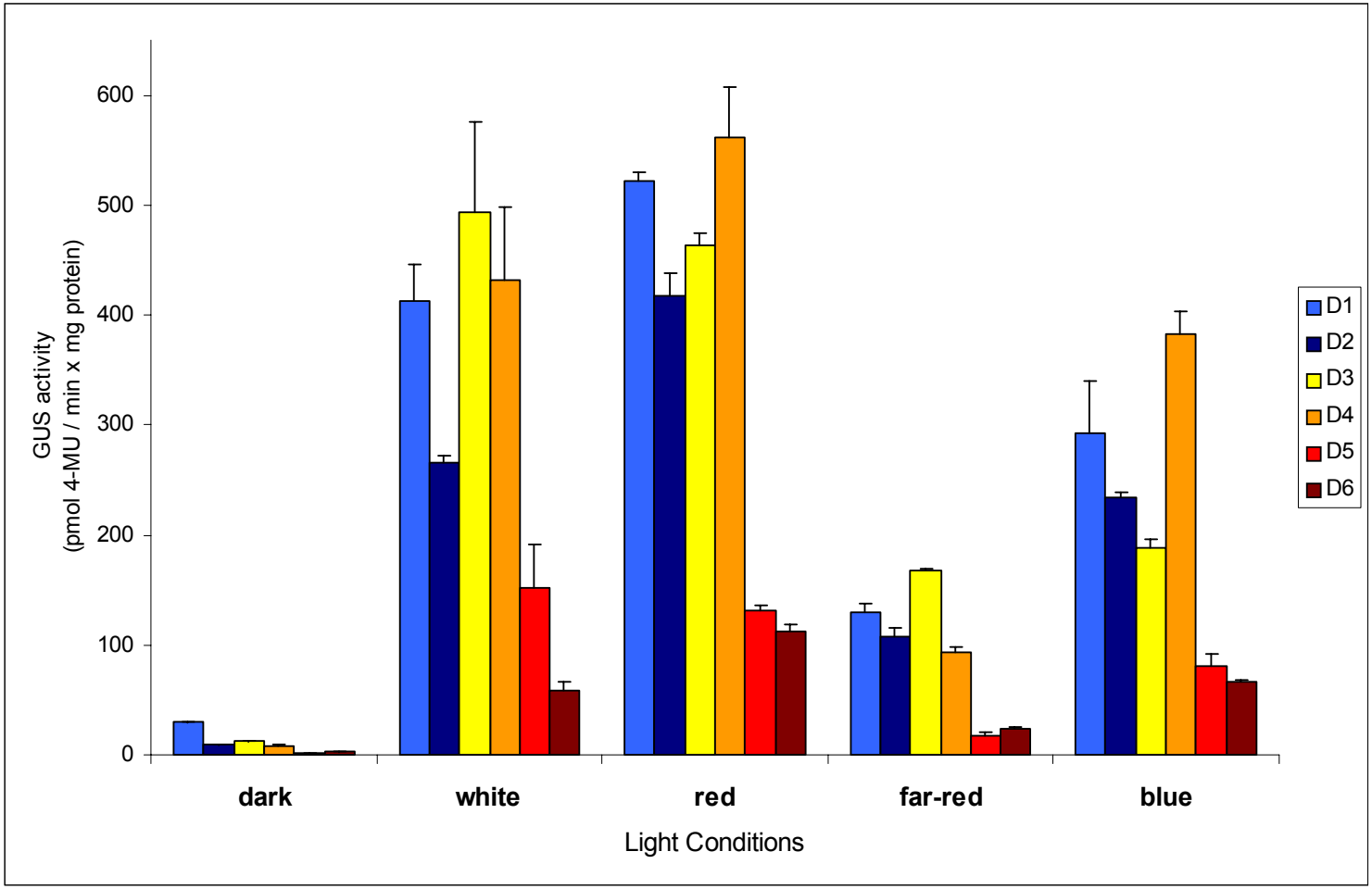


Figure 15: GUS activity of transgenic *Arabidopsis* plants carrying different length HEMA1 promoter:GUS reporter gene fusions in response to continuous light of different wavelengths

Graphical depiction of results shown in Tab. 5. Transgenic *Arabidopsis* plants carrying *HEMA1* promoter deletion:*gusA* constructs were grown in seven days continuous dark, white, red, far-red or blue light. Protein extracts were prepared and fluorometric GUS assays were performed. GUS activity was measured as rate of enzymatic 4-methyl-umbelliferone (4-MU) production per min and normalized to the total protein content of each extract (error bars show standard error).

Further experiments will have to be performed to confirm the involvement of these photoreceptors. For example, the use of phytochrome deficient mutants and cryptochrome/phototropin mutants may help elucidate the specific role of photoreceptors in the light response of *HEMA1*. After the completion of the practical part of this study, various photoreceptor mutants were tested for induction of *HEMA1* expression and showed the involvement of phytochrome and cryptochrome signaling pathways (McCormac and Terry, 2002).

4.1.8. PP1/PP2A-mediated Regulation of the *HEMA1* Promoter

Plant phytochromes have an intramolecular Ser/Thr kinase activity (Yeh and Lagarias, 1998) and the phytochrome phyA was described as a phosphoprotein *in vivo* with two mapped phosphorylation sites (Lapko *et al.*, 1999). Downstream substrates for kinase cascades are being investigated and so far a blue light photoreceptor (CRY1) (Ahmad *et al.*, 1998) as well as new proteins like PIF3, a phytochrome-interacting factor (Ni *et al.*, 1998) have been implicated. Also, blue light activated autophosphorylation of another blue light receptor, phototropin1 (Phot1), through a serine/threonine kinase domain has been shown and a link between blue-light mediated phototropism and photophosphorylation was observed (reviewed in Sullivan and Deng, 2003).

With these implications for a role of reversible protein phosphorylation in light regulation, the question arose if phosphatases could have an influence on the light response seen with the *HEMA1* promoter. Prominent elements in eukaryotic signal transduction are the protein phosphatases 1 and 2A (PP1/2A). To test their possible involvement, transgenic plants carrying different *HEMA1* promoter deletion:*gusA* constructs were grown for ten days in the light (16 h light and 8 h dark day cycles) or dark in the presence of a known phosphatase inhibitor. Cantharidin, an inhibitor of both protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A), can be used to distinguish between the two phosphatases by their different IC_{50} values needed for inhibition. The IC_{50} value of purified PP2A is 0.16 μM, whereas the IC_{50} value for PP1 lies at 1.7 μM. However, these inhibition values have been determined with purified enzymes *in vitro*. Inhibition in bioassays *in vivo* is generally observed at much higher concentrations and thus does not provide a clear distinction between PP1 and PP2A. The concentration of cantharidin selected for the following experiment was chosen due to the known inhibition of a PP2A dependent process like root elongation at the concentration of 5 μM (Deruère *et al*., 1999).

Approximately 100 seeds of several homozygote lines containing different *HEMA1* promoter deletion:*gusA* constructs were each plated onto duplicate plates containing 5 μM cantharidin. The plates were vernalized in the dark at 4° C for three days, followed by a four hour white light

treatment and the seedlings were then grown for ten days either in the dark or in white light (16 h light and 8 h dark day cycles). The complete seedlings were harvested, frozen in liquid nitrogen and protein extracts were prepared. The protein concentration of the extracts was determined and GUS activity was measured as described in Materials and Methods. GUS activity was determined as rate of enzymatic 4-methyl-umbelliferone (4-MU) production per minute and normalized to the total protein content of each extract. Several independent homozygote transgenic lines were tested for each *HEMA1* promoter deletion construct and a representative result from one typical transgenic line for each construct is shown in Table 6. Fig. 16 shows a graphical depiction of the results. The results already described under 4.1.6. were added to allow the comparison of levels of GUS activity in the presence and absence of phosphatase inhibitor.

In the presence of cantharidin, light-grown transgenic plants carrying *HEMA1* promoter deletion constructs showed GUS reporter gene expression that was at about the same level than in the absence of phosphatase inhibitor. The overall observed pattern of expression between the different length *HEMA1* promoter deletions was also very similar between plants grown in the presence or absence of cantharidin. Transgenic plants grown on plates containing cantharidin showed the highest level of GUS reporter gene expression with the longest promoter deletion D1 and the lowest expression with the shortest *HEMA1* promoter construct D6. The observed absolute values for these two constructs were nearly identical in the presence and absence of phosphatase inhibitor. Both in the presence and absence of cantharidin, the second promoter deletion construct D2 showed an abrupt drop in the level of GUS expression to 37% and 22%, respectively, of the level of D1. In both cases the level of GUS expression recovered with the next *HEMA1* promoter deletion construct D3, before dropping down again with D4. The only observable difference between light-grown plants in the presence and absence of cantharidin was observed for *HEMA1* promoter deletion construct D5. Despite a high degree of variation among transgenic lines carrying this particular construct, the level of achieved GUS reporter gene expression was never higher than about 40% of the value for D1 (data not shown). However, in the presence of cantharidin light-grown transgenic plants carrying construct D5 showed 80% of the reporter gene expression level of D1.

Table 6: GUS activity of transgenic *Arabidopsis* plants carrying *HEMA1* promoter deletion:GUS constructs in the presence and absence of PP1/PP2A inhibitor cantharidin

with cantharidin			
	10 days light	**10 days dark**	fold induction between expression in dark and in light
Promoter Deletion Construct	average GUS activity [1] (pmol 4-MU mg protein^{-1} min^{-1})	average GUS activity [1] (pmol 4-MU mg protein^{-1} min^{-1})	
D1	504.4 +/- 29.1	156.0 +/- 21.2	**3**
D2	186.5 +/- 43.3	65.9 +/- 11.5	**3**
D3	280.6 +/- 28.1	108.0 +/- 14.7	**3**
D4	122.4 +/- 12.6	30.6 +/- 3.1	**4**
D5	388.0 +/- 44.5	156.9 +/- 19.5	**2**
D6	44.5 +/- 8.3	6.6 +/- 2.4	**7**

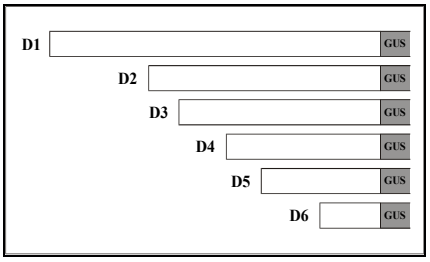
without cantharidin [2]			
	10 days light	**10 days dark**	fold induction between expression in dark and in light
Promoter Deletion Construct	average GUS activity (pmol 4-MU mg protein^{-1} min^{-1})	average GUS activity (pmol 4-MU mg protein^{-1} min^{-1})	
D1	485.2 +/- 42.5	25.1 +/- 1.2	**19**
D2	108.0 +/- 11.6	9.3 +/- 0.3	**12**
D3	221.5 +/- 1.5	15.8 +/- 1.5	**14**
D4	78.4 +/- 8.1	3.9 +/- 0.2	**20**
D5	117.1 +/- 3.4	25.3 +/- 0.9	**5**
D6	53.7 +/- 3.0	5.8 +/- 0.4	**9**

[1] GUS activity was measured as rate of enzymatic 4-methyl-umbelliferone (4-MU) production per min and normalized to the total protein content of each extract. Measurements were taken in duplicates or quadruplicates and averages and their standard error (SE) were calculated. [2] Values of GUS activities as determined in 4.1.6.

Table 7: Difference of GUS activity in the absence and presence of cantharidin

Percent difference between GUS activity in the absence and presence of cantharidin						
	D1	**D2**	**D3**	**D4**	**D5**	**D6**
expression in light	96%	58%	79%	64%	30%	121%
expression in dark	16%	14%	9%	13%	16%	88%

a.



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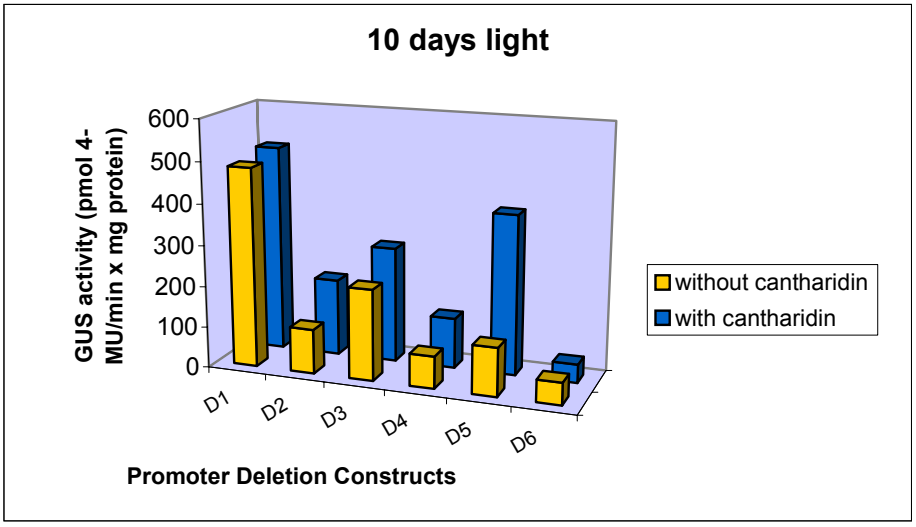

c.

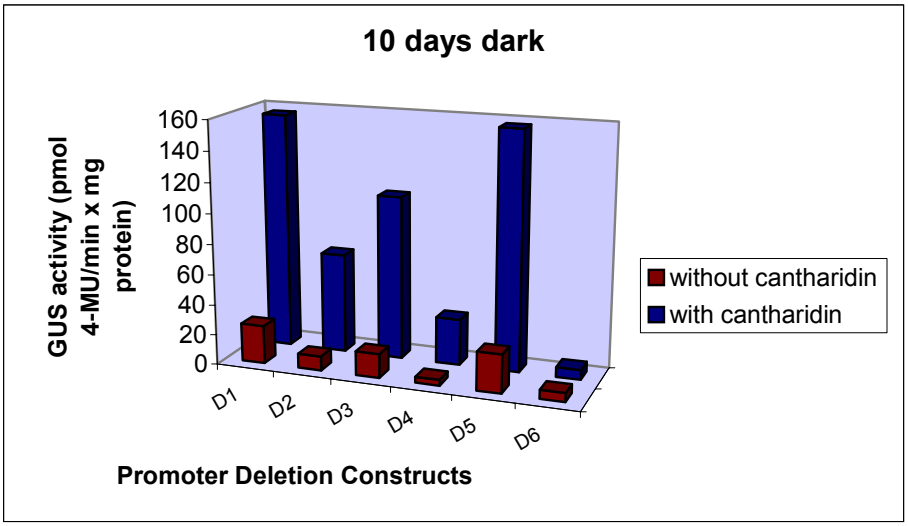

Figure 16: GUS activity of cell-free extracts prepared from transgenic *Arabidopsis* plants in the presence and absence of cantharidin

Graphical depiction of results shown in Tab. 6. Transgenic *Arabidopsis* plants carrying *HEMA1* promoter deletion:GUS constructs were grown in ten days light or ten days dark in the presences of protein phosphatase 1 and 2A inhibitor cantharidin. Protein extracts were prepared and fluorometric GUS assays were performed. GUS activity was measured as rate of enzymatic 4-methyl-umbelliferone (4-MU) production per min and normalized to the total protein content of each extract.

a. Schematic of *HEMA1* promoter deletion:GUS constructs. b. Comparison of GUS activities of transgenic lines grown in the absence and presence of cantharidin for ten days in light carrying different *HEMA1* promoter deletion:GUS constructs. c. Comparison of GUS activities of transgenic lines grown in the absence and presence of cantharidin for ten days in the dark carrying different *HEMA1* promoter deletion:GUS constructs.

This could indicate the presence of a responsive element within the sequence between -960 bp and -451 bp upstream of the translation start site that is directly or indirectly down-regulated by the phosphatase activity of PP1 or PP2A.

A significant difference between the presence and absence of cantharidin could be noted with dark-grown transgenic plants. With the exception of the shortest *HEMA1* promoter deletion construct D6, plants carrying all other promoter deletion constructs showed a considerably higher level of GUS reporter gene expression in the presence of cantharidin compared to the absence of phosphatase inhibitor. The pattern of expression between the different *HEMA1* promoter deletion:*gusA* constructs mimicked the pattern seen in light grown plants. The highest GUS activity was seen with the longest construct D1, followed by a drop in expression with D2 and a recovery of GUS levels with D3. The expression then dropped again with D4 before showing a level of expression equal to the D1-level with D5. The observed reporter gene expression from the shortest *HEMA1* promoter deletion construct D6 was the lowest value of all and matched the value measured in the absence of cantharidin. The overall level of GUS expression shown by constructs D1 to D5 were about 6 - 8 fold higher in the presence of cantharidin than in the absence of phosphatase inhibitor. This lowered the values for induction between reporter activity in dark-grown plants and light-grown plants to three to seven fold compared to 5 - 20 fold in the absence of cantharidin.

Overall, it was concluded that the positive light response of the *HEMA1* promoter seems to be generally independent of the activity of protein phosphatase 1 or 2A as similar levels of reporter gene expressions were observed in the presence and absence of a specific phosphatase inhibitor. However, initially it appears that the basal, light-independent expression driven by the *HEMA1* promoter seems to be influenced by the activity of protein phosphatases. The lack of dephosphorylation of an unknown protein either directly or indirectly causes the *HEMA1* promoter expression to be up-regulated. Alternatively, one could argue for a phosphatase-dependent regulatory mechanism responsible for the repression of *HEMA1* expression in the dark.

The results of this experiment indicated the possibility of an element within the region of -960 bp and -451 bp in the *HEMA1* upstream sequence that shows a strong response to PP1 or PP2A in dark-grown transgenic plants as well as a weaker reaction even in light-grown plants.

Further experiments would have to be done to substantiate the implication of PP1/PP2A involvement in the down-regulation of the transcription of *HEMA1* and to determine which of the two Ser/Thr specific protein phosphatases is indeed having the effect.

4.1.9. Regulatory Model of the *Arabidopsis thaliana HEMA1* Promoter

The experiments described in this part of this study have shown that the *Arabidopsis HEMA1* promoter shows various different modes of light-dependent responses.

A model summarizing the results of the promoter analysis of the *Arabidopsis thaliana HEMA1* gene is depicted in Fig. 17. The shortest *HEMA1* promoter fragment D6 is sufficient to cause the observed positive light response. Red light, most likely mediated by the phytochrome system, and blue light significantly induce *HEMA1* expression from this promoter element. The D6 fragment contains some potential promoter elements proposed to be involved in light regulation (I-box core, GATA motif). Additional potential regulatory elements for currently unknown stimuli conserved between the promoters of *HEMA1*, *HEMA2* and *GSA1* are also localized within the sequence of D6.

Repression of *HEMA1* expression in the dark (negative light response) involving PP1/PP2A dephosphorylation is mediated by the D5 part of the promoter sequence. All other areas of the *HEMA1* promoter besides the deletion D2 contribute to the overall promoter strength. Elements within the D2 sequence clearly repress *HEMA1* promoter activity.

These finding provide first insights into the complex regulatory scenario of *HEMA1* expression. Clearly, further experiments using regulatory mutants and a screen for the response to additional intra- and extracellular parameters (for example heme content, energy status of the cells, iron accessibility, oxygen stress, osmolarity etc.) can shed light onto involved parameters, factors and their coordinated interplay.

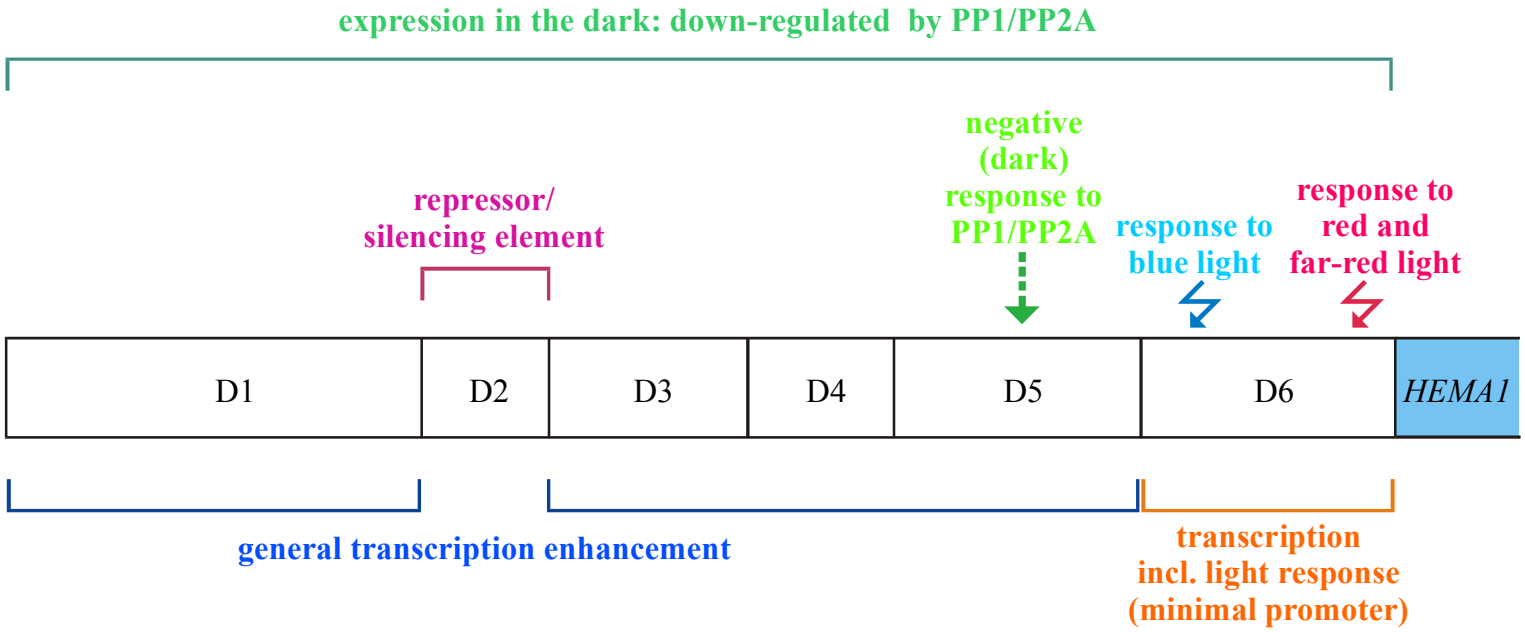

Figure 17: Regulatory model of *Arabidopsis thaliana HEMA1* gene promoter

4.2. PP2A-mediated Signal Transduction in Plants

As shown in 4.1., both light and protein phosphatases (PP1 or PP2A) are independently involved in the regulation of *HEMA1*. Recent studies revealed a link between blue-light mediated phototropism and photophosphorylation (reviewed in Sullivan and Deng, 2003). It has furthermore been shown that phototropin1 (Phot1) has a serine/threonine kinase activity. Moreover, plant phytochromes that perceive red and far-red light have intramolecular Ser/Thr kinase activity (Yeh and Lagarias, 1998). Protein phosphatase 1 and 2A are serine/threonine phosphatases and therefore their involvement as possible antagonists in light regulation signal transduction cascades would be feasible. However, so far no reports have linked PP1/PP2A to light regulation in plants (see recent review in Luan, 2003).

To gain further knowledge about signal transduction cascades involving PP2A in *Arabidopsis*, an attempt was made to identify possible interaction partners of PP2A.

4.2.1. Identification of PP2A Interaction Partners

Protein Phosphatase 2A has been implicated in many pathways as different as cell cycle regulation, cell morphology and development in many organisms (Janssens and Goris, 2001). In plants, PP2A was shown to be involved in processes ranging from cold response to carbon and nitrogen metabolism. The enzyme system seems to be crucial for plant developmental processes and hormone signal transduction like the regulation of auxin transport (Monroy *et al.*, 1998, Garbers *et al.*, 1996, recently reviewed in Luan, 2003). As described in greater detail in 2.2.3., PP2A is a trimeric enzyme and in *A. thaliana* a multitude of catalytic C-subunits and regulatory subunits lending specificity to the holoenzyme have been described (Luan, 2003).

At the time of this study, five catalytic subunits of protein phosphatase 2A had been described (Ariño *et al*., 1993; Casamayor *et al*., 1994; Stamey and Rundle, 1996). These subunits, labeled C-1 to C-5, are divided into 2 subfamilies based on the degree of homology of their amino acid sequences with C-1, C-2 and C-5 in one subgroup and C-3 and C-4 comprising the second subgroup (Pérez-Callejón *et al.,* 1998).

Since that time, several closely related proteins have been described (Kim *et al.*, 2002). However, none of these proteins fit into the described two subfamilies which is clearly visible from the pair-wise comparison of all currently known catalytic subunits of PP2A and closely related proteins shown in Tab 8. All catalytic subunits of PP2A show an identity of 93 - 99% within their subfamilies and a 78 - 79 % identity between the subfamilies. The degree of

Table 8: Pair-wise comparison of catalytic subunits of PP2A and closely related proteins from *A. thaliana* and *H. sapiens*.

Protein sequences were obtained by searching the *Arabidopsis* database at mips (Munich Information Center for Protein Sequences - http://mips.gsf.de) and at TAIR (The Arabidopsis Information Resource - http://www.arabidopsis.org). The human protein sequence was found by performing a BLAST search at NCBI (National Center for Biotechnology Information - http://www.ncbi.nlm.nih.gov) with the protein sequence of PP2Ac-2 and selecting the closest human homologue. Pair-wise protein sequence alignments were performed using http://searchlauncher.bcm.tmc.edu (using BLAST 2 located at NCBI). Values in the lower left half of the tables are degrees of identities (labelled in red), whereas values in the upper right half of the tables denote the degree of homology (labelled in green).

		At1g59830	**At1g10430**	**At2g42500**	**At3g58500**	**At1g69960**	**At4g26720**	**At5g55260**	**At3g19980**	**At1g50370**	**At2g39840**	**CAG33698**
		PP2Ac-1	PP2Ac-2	PP2Ac-3	PP2Ac-4	PP2Ac-5	PPX-1	PPX-2	AtFyPP3	AtFyPP1	PP1-4	PPP2CA-Hu (H. sapiens)
At1g59830	PP2Ac-1	-	301/306 (98%)	269/301 (88%)	268/301 (88%)	296/305 (96%)	247/303 (81%)	245/303 (80%)	224/302 (73%)	224/302 (73%)	195/295 (66%)	276/301 (91%)
At1g10430	PP2Ac-2	297/306 (97%)	-	270/301 (89%)	269/301 (88%)	297/305 (96%)	248/303 (81%)	246/303 (81%)	224/302 (73%)	224/302 (73%)	197/295 (66%)	278/301 (91%)
At2g42500	PP2Ac-3	239/301 (79%)	238/301 (79%)	-	311/313 (99%)	273/304 (88%)	249/304 (81%)	245/303 (80%)	221/303 (72%)	222/303 (72%)	187/274 (67%)	271/303 (88%)
At3g58500	PP2Ac-4	238/301 (79%)	239/301 (79%)	307/313 (98%)	-	272/304 (89%)	248/304 (81%)	244/303 (79%)	221/303 (72%)	222/303 (72%)	186/274 (67%)	271/303 (88%)
At1g69960	PP2Ac-5	286/305 (93%)	285/305 (93%)	240/304 (78%)	241/304 (79%)	-	247/303 (81%)	246/303 (80%)	226/302 (74%)	226/302 (74%)	187/276 (67%)	277/303 (90%)
At4g26720	PPX-1	198/303 (65%)	198/303 (65%)	203/304 (66%)	202/304 (66%)	197/303 (65%)	-	295/305 (95%)	238/306 (77%)	240/306 (77%)	201/297 (67%)	246/304 (80%)
At5g55260	PPX-2	199/303 (65%)	197/303 (65%)	197/303 (65%)	196/303 (64%)	199/303 (65%)	286/305 (93%)	-	232/304 (75%)	234/304 (76%)	194/282 (67%)	244/303 (79%)
At3g19980	AtFyPP3	172/302 (56%)	173/302 (57%)	172/303 (56%)	172/303 (56%)	173/302 (57%)	188/306 (61%)	181/304 (59%)	-	302/303 (99%)	182/262 (68%)	222/302 (73%)
At1g50370	AtFyPP1	170/302 (56%)	171/302 (56%)	171/303 (56%)	171/303 (56%)	171/302 (56%)	188/306 (61%)	181/304 (59%)	300/303 (99%)	-	183/262 (69%)	222/302 (72%)
At2g39840	PP1-4	136/295 (46%)	138/295 (46%)	131/274 (47%)	129/274 (47%)	134/276 (48%)	129/297 (43%)	132/282 (46%)	122/262 (46%)	121/262 (46%)	-	186/275 (66%)
CAG33698	PPP2CA-Hu (H. sapiens)	241/301 (80%)	240/301 (79%)	244/303 (80%)	244/303 (80%)	242/303 (79%)	198/304 (65%)	196/303 (64%)	173/302 (57%)	171/302 (56%)	140/275 (50%)	-

identity to the described other catalytic subunits is significantly lower with values of 64 - 66% to PPX-1 and PPX-2, 56 - 57% to AtFyPP1/AtFyPP3 and only 46 - 48% to PP1-4. Interestingly, the identity of the C-1 to C-5 proteins to a human catalytic subunit of PP2A is with 79-80% higher than to the other catalytic subunits from *Arabidopsis.* These different degrees of identity are also reflected by the nomenclature of all *Arabidopsis* subunits since none has been named a PP2A C-subunit.

Expression studies of the genes encoding PP2A catalytic subunits in *Arabidopsis* had been performed prior to this study and showed a similar level of mRNA expression in all tissues. However, slightly different levels mRNA were reported in adult plants compared to seedlings with C-1 showing the highest level of expression in adult plants and C-3 showing the highest level of expression in seedlings (Ariño *et al*., 1993; Pérez-Callejón *et al.*, 1993).

By recognizing potential substrates for dephosphorylation, it was attempted to identify the involvement of protein phosphatase 2A in different aspects of cellular regulation and signal transduction. For this purpose, catalytic subunits from both subfamilies within the PP2A catalytic subunits should be considered. Following the expression studies described in the literature, C-1 and C-3 represented good candidates. In order to find possible targets for dephosphorylation by PP2A in *A. thaliana* and therefore place PP2A in new signal transduction pathways, the two described C-subunits (C-1 and C-3) were selected and used as bait proteins in a yeast two-hybrid screen.

4.2.2. Experimental Rationale: Description of Yeast Two-Hybrid System

The yeast two-hybrid system was first described by Fields and Song in 1989 as a novel genetic system to detect protein-protein interactions. Even though research for many organisms has since moved into the post-genomic era, the original yeast two-hybrid system and its modifications are still considered powerful tools to determine the network of interactions between proteins expressed in a cell (Causier and Davies, 2002). In contrast to biochemical approaches such as co-immuno-precipitations and affinity chromatography, the two-hybrid systems allows for examination of proteins expressed *in vivo*, therefore avoiding problems due to a lack of post-translational modifications, incorrect folding or protein instability due to buffer conditions.

The basic principle underlying these systems is the separation of a discrete DNA-binding and transcriptional activation domain. These domains are fused to a bait protein and potential interacting proteins, respectively. If interaction occurs between bait and prey proteins, the

activation and binding domains will be brought into close proximity to each other and the resulting transcription of reporter genes in a suitable yeast strain can be detected.

The system used for this study was obtained from the Arabidopsis Biological Resource Center (ABRC) and is described in Fig. 18.

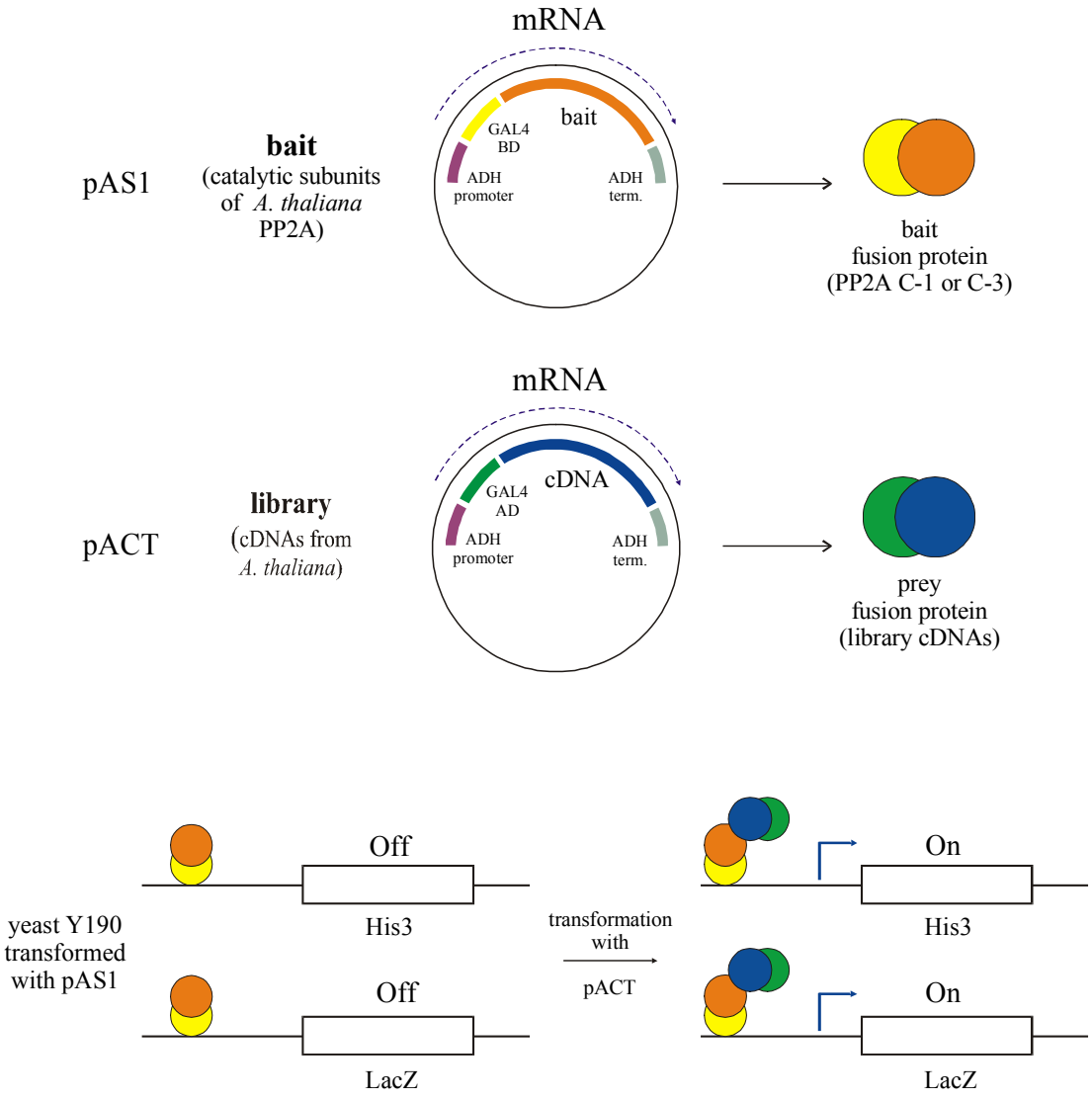

Figure 18: Schematic representation of the yeast two-hybrid system employed in this study

The cDNAs for *A. thaliana* PP2A C-1 and C-3 subunits were fused in-frame to the GAL4 DNA binding domain (GAL4 BD) in the bait vector pAS1 (Durfee *et al.*, 1993). Two different cDNA libraries were obtained from the Arabidopsis Biological Resource Center (NSF/DOE/USDA Collaborative research in Plant Biology Program, Research Collaboration Group in Plant Protein Phosphorylation, USDA 92-37105-7675; Kim, J. and Theologis, A.). The library cDNAs were constructed using the plasmid pACT (Durfee *et al.*, 1993) to produce a fusion protein containing the GAL4 transcription activation domain (GAL4 AD). After sequential transformation with both plasmids into the yeast strain Y190 (Harper *et al.*, 1993) interaction of expressed fusion proteins reconstitutes an active transcription factor which specifically binds to promoter elements upstream of the two reporter genes. This activated their expression and allows for selection on yeast medium lacking histidine and produces a blue coloring of colonies in yeast medium containing X-gal.

In this work, two PP2A C-subunits of *Arabidopsis* were used as bait proteins to find possible interacting proteins from two different cDNA libraries. The catalytic subunit C-1 which was reported to be the major mRNA species in adult plants (Pérez-Callejón *et al.*, 1993) was searched against proteins expressed from a cDNA library from mature leafs and roots. The catalytic subunit C-3 which showed highest mRNA expression in seedlings (Ariño *et al*., 1993) was used with a cDNA library developed from 3 day-old etiolated seedlings. Both libraries were obtained cloned in λ-phage. The conversion of the λ-ACT cDNA library into the pACT plasmid library was performed by Dr. Weizhong Chang and plasmid DNA was a kind gift. The vectors used were developed by Dr. Steve Elledge and are described in Durfee *et al.*, 1993, whereas the yeast strain Y190 was described in Harper *et al.*, 1993. The cDNA libraries were constructed by members of the NSF/DOE/USDA Collaborative research in Plant Biology Program, Research Collaboration Group in Plant Protein Phosphorylation (USDA 92-37105-7675) and Dr. J. Kim. and Dr. A. Theologis. The positive control used for this screen was described by Fields and Song, 1989. It contains the bait protein SFN1 which was shown to interact with SNF4, thus activating reporter gene expression.

4.2.3. Construction of Bait Vector containing PP2A C-1 and C-3 Catalytic Subunits

The catalytic subunits C-1 and C-3 from *A. thaliana* were selected to use as bait proteins in a yeast two-hybrid screen searching cDNA libraries prepared from mature leafs and roots and from three days old etiolated seedlings.

Oligonucleotides for PCR-cloning were designed from published cDNA sequences of the *Arabidopsis* PP2A catalytic subunits. Special care was taken to match the subunits C-1 and C-3 specifically and avoiding primer design that would select the closely related catalytic subunits C-2, C-4, C-5 or the described PPX. All forward primers were designed to enable in-frame subcloning of the bait protein into the yeast vector ensuring the proper expression of the fusion protein for the two-hybrid system screen.

The appropriate PP2A C-1 and C-3 subunit cDNA was obtained through a reverse transcription reaction from RNA isolated from mature leafs (gift from Dr. A.M. Kumar) and from whole seedlings. The resulting first strands were then used as templates for PCR reactions using the primers designed for the two selected catalytic subunits of PP2A.

PCR products of the expected sizes of 950 bases for C-1 and 1070 bases for C-3 were observed through agarose gel electrophoresis by comparing the DNA fragments to DNA markers of known sizes (data not shown). A restriction site map was generated using the published cDNA sequences. Restriction analysis was used to confirm the identity of the PCR products. Restriction enzymes were selected that showed cutting sites in C-1 or C-3, but not in members of the respective subfamily of catalytic subunits of PP2A in *Arabidopsis*. Fig. 19a shows a schematic of the restriction maps for the PP2A catalytic subunits C-1 and C-3 demonstrating the expected fragments in restriction analysis considering the cloned fragments are not C-2, C-4 or C-5. This analysis showed only fragments of expected sizes, demonstrating that the amplified PCR products were indeed C-1 and C-3 (data not shown).

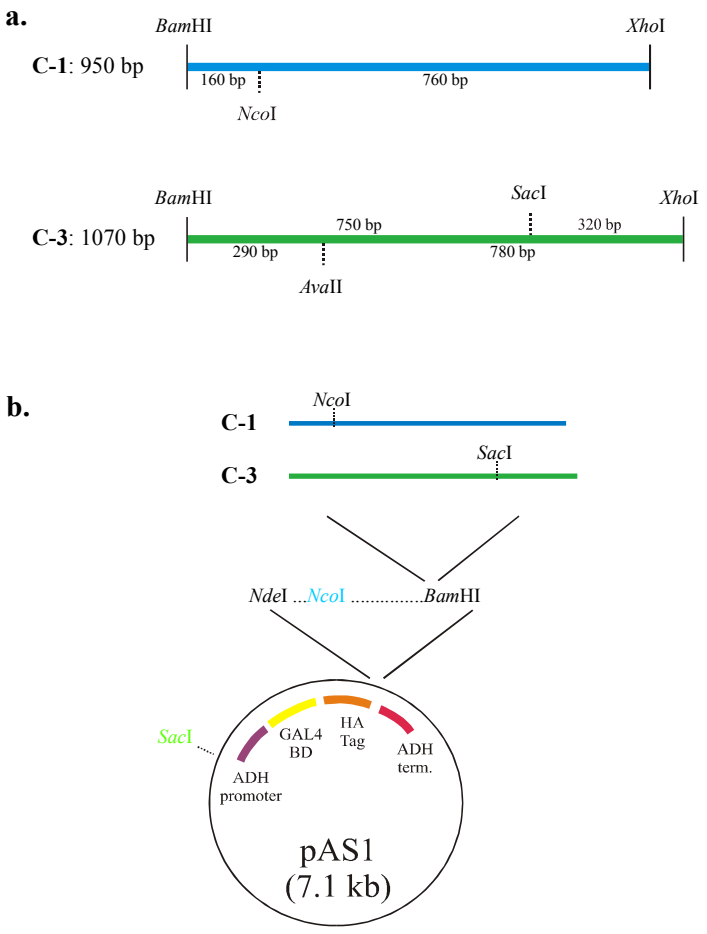

Figure 19: Restriction maps of *A. thaliana* PP2A C-1 and C-3 subunit cDNAs
a. Map of the catalytic subunits C-1 and C-3
b. Map of yeast vector pAS1: Restriction sites used for subcloning of pAS1-C1 and pAS1-C3

In order to facilitate the successful subcloning of the obtained PCR fragments into the yeast bait vector pAS1, a subcloning step into the *E.coli* TA vector was performed first. Plasmid DNA was isolated and restriction digests were performed to confirm the presence of plasmid with inserts of the expected size and the correct restriction sites (data not shown). Colonies carrying plasmids with the correct inserts were selected and isolated plasmid DNA was used for further subcloning.
The C-1 and C-3 cDNA inserts were released from the respective TA-vector by *Bam*HI digest, ligated into linearized binary bait vector pAS1 and *E. coli* JS5 cells were transformed. Restriction digests were performed and colonies carrying plasmids with inserts of the expected size and at the correct orientation were selected (data not shown). Further plasmid DNA was isolated and used for subsequent transformations into yeast.

4.2.4. Yeast Two-Hybrid Screen for PP2A Subunit C-1 and C-3 Interaction Partners

The yeast two-hybrid screen was performed based on Fields and Song, 1989 with modifications as described by Harper *et al.*, 1993 and Durfee *et al.*, 1993 and Finley and Brent, 1995.
A sequential transformation of plasmids was performed following protocols described in Material and Methods. A flow chart outlining the steps of the screening is shown in Fig. 20.

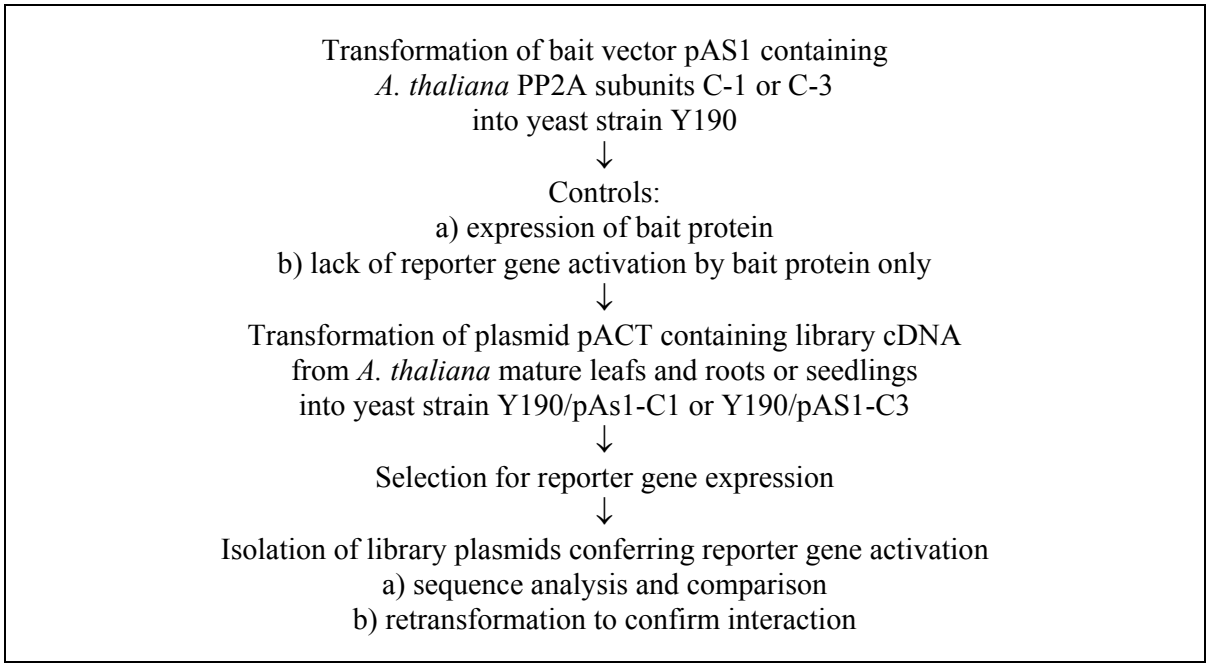

Figure 20: Two-hybrid screen outline
A flow chart describing the steps employed in carrying out the two-hybrid screen for interaction partners of the PP2A catalytic subunits C-1 and C-3.

4.2.4.1. Transformation of C-1 and C-3 Bait Vector into Yeast Strain Y190

A. thaliana PP2A catalytic subunits C-1 and C-3 cDNAs contained in the binary vector pAS1 were transformed into the yeast strain Y190 (Harper *et al.*, 1993 and Durfee *et al.*, 1993). The strain contains two chromosomally located reporter genes whose expression is regulated by the yeast transcription factor Gal4. One is the *E. coli lacZ* gene under the *GAL1* promoter control. The second is the selection marker *HIS3* whose gene product allows for histidine prototrophy of the histidine auxotrophic yeast strain Y190 at very low levels and therefore enables a very high sensitivity in screening. However, even though the endogenous *gal4* transcription factor gene and its negative regulator gene *gal80* have been deleted in Y190, residual expression of *HIS3* is sufficient for growth on media without histidine. This can be overcome by growing cells in the presence of 25 mM 3-aminotriazole (3-AT), a chemical inhibitor of the *HIS3* gene product. The activation of both reporter genes should only occur when the Gal4 DNA-binding domain fusion protein (bait protein) comes into close proximity to the Gal4 transcriptional activation domain of the library encoded fusion protein (prey protein).

Additionally, Y190 is auxotroph for tryptophan and leucine resulting in the inability of cells to grow on media lacking these amino acids. The bait plasmid pAS1 encodes the *TRP1* gene which allows for growth on media lacking tryptophan, whereas the *LEU2* gene localized on the library plasmid pACT enables leucine prototrophy. This means that Y190 cells cannot grow on media lacking tryptophan, leucine or histidine in the presence of 3-AT nor can *lacZ* activation occur. Controls have to be performed to check for both expression of a bait protein after transformation and the lack of reporter gene activation by the expressed bait protein without interaction. Only thereby it can be ensured that following transformation of Y190 with the bait plasmid growth on media lacking tryptophan is possible, however no activation of the reporter genes occurs.

4.2.4.2. Determining Production of the C-1 and C-3 Bait Proteins in Yeast

The yeast strain Y190 had been transformed with bait vector pAS1 containing either the genes encoding for the *A. thaliana* PP2A C-1 or C-3 subunit. Following this transformation, the expression of the bait proteins was tested through Western blot analysis.

Protein extracts were prepared from Y190, Y190 transformed with pAS1-C1 or pAS1-C3 and the positive control for the two-hybrid screen, SNF1/SNF4, using two different protocols (labeled E-1 and E-2). The protein extracts were analyzed by Western blot using two different antibodies (Fig. 21).

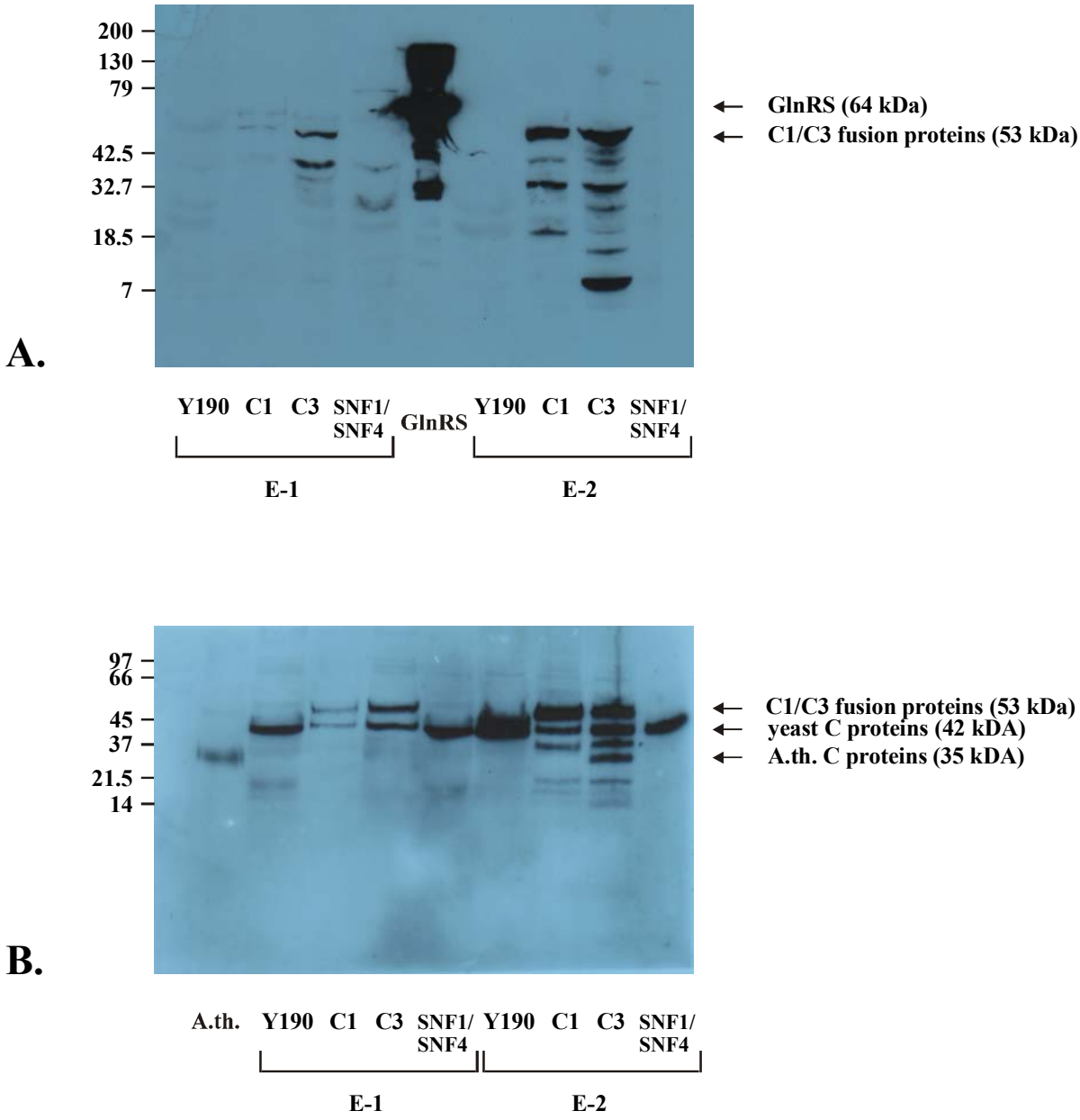

Figure 21: Western blot analysis determining production of the bait fusion protein

Protein extracts were prepared using two different protocols (labeled E-1 and E-2) from yeast Y190, Y190 transformed with pAS1-C1 and pAS1-C3 and the positive control for the two-hybrid screen, SNF1/SNF4. The protein extracts were separated by SDS-PAGE and Western Blot analysis was performed. Antibodies against different parts of the expected fusion proteins were used.

A. Anti-HA-antibody was used to detect HA epitope tags of fusion proteins; HA-GlnRS was added as positive control. Bands of expected molecular weight were observed in the extracts of Y190/pAS1-C1 and Y190/pAS1-C3.

B. Anti-PP2A-C-antibody was used to detect PP2A catalytic subunit fusion proteins; *A. thaliana* wild-type plant extract was added as positive control. Bands of expected molecular weight were observed for the fusion proteins in the extracts of Y190/pAS1-C1 and Y190/pAS1-C3.

Relative molecular masses (x 10^3) of marker proteins are indicated.

As the bait plasmid pAS1 contains sequence information to add a HA epitope tag to the fusion protein, an anti-HA-antibody was used for the Western Blot shown in section A of Fig. 21. As positive control for the antibody, a partially purified GlnRS protein with HA tag (gift from Dr. Jinhua Liu) was included on the gel with an expected molecular weight of 64 kDa. The expected molecular weight for the recombinant *A. thaliana* PP2A catalytic subunits C-1 and C-3 are 34.9 kDa and 35.8 kDa, respectively. The plasmid pAS1 encodes 147 amino acids of the GAL4 DNA-binding domain which adds an estimated 18 kDa to the fusion protein. After incubation with the anti-HA antibody, the blot showed a main protein band of about 64 kDa for the HA-GlnRS for the positive control. Both the extracts from Y190/pAS1-C1 and Y190/pAS1-C3 showed bands of the expected molecular weight of 53 kDa for the fusion proteins. Additionally, bands indicating various degradation products were visible which are most likely due to the different methods used to prepare the protein extract. No obvious protein bands were seen in the extracts from Y190 and the two-hybrid positive control SNF1/SNF4.

For the Western blot shown in section B of Fig. 21, an anti-C-antibody was used. This antibody was produced in rabbits against a carboxy peptide of the PP2A catalytic subunit of *Xenopus laevis*. Due to the high degree of sequence similarity and conservation of PP2A catalytic subunits across different species (Favre *et al.*, 1994), it is expected that the antibody will react with the catalytic subunits of *A. thaliana* wild-type PP2A, yeast PP2A and the contained *A. thaliana* subunits C-1 and C-3 within the bait fusion protein. The expected molecular weight of the described *S. cerevisiae* PP2A catalytic subunits PPH21 and PPH22 is around 42 kDa, whereas the *Arabidopsis* wild-type subunits (C-1 through C-5) are expected to be around 35 kDa. Additionally to the yeast extracts from Y190, Y190/pAS1-C1, Y190/pAS1-C3 and the positive control SNF1/SNF4, *A. thaliana* plant extract (gift from Dr. J. Deruère) was included in the analysis. After incubation with the anti-C-antibody, the blot showed a band of the expected size for the *A. thaliana* wild-type catalytic subunits. Also, the yeast extracts without pAS1 fusion protein (Y190 and SNF1/SNF4) showed a protein band at the expected position for the yeast catalytic subunits. The extracts from Y190/pAS1-C1 and Y190/pAS1-C3 showed the same sized protein band for the yeast catalytic subunits. Additionally, a band of the expected size for the fusion protein of C-1 or C-3 and the GAL4 DNA-binding domain was present on the Western blot. In summary, both blots showed antibody recognition for proteins of the expected molecular masses for the fusion protein of the GAL4 DNA-binding domain and the *A. thaliana* PP2A catalytic subunits C-1 and C-3.

4.2.4.3. Lack of Reporter Gene Expression by Bait Protein only

Appropriate controls to test the bait plasmid for the use in screening had to be performed to ensure that no reporter gene activation was induced by the presence of bait protein only.

As shown in Fig. 22, the original Y190 strain, Y190 transformed with pAS1 containing the *A. thaliana* PP2A C-1 or C-3 subunit and Y190 containing a positive control for the two-hybrid screen were tested on different media plates. The positive control produces the bait protein SFN1 which was shown to interact with SNF4, thus activating reporter gene expression (Fields and Song, 1989). Four colonies of each strain were patched onto plates containing different media components and grown at 30° C for two days.

All strains were able to grow on full yeast media YPD (plate a) showing that all transformed cells were viable and showed similar growth compared to the original strain Y190. Plate b contained media lacking tryptophan to test for the presence of bait plasmid pAS1. The original strain Y190 being tryptophan auxotroph was unable to grow. However, the positive control, Y190/pAS1-C1 and Y190/pAS1-C3 sustained full growth on this media lacking tryptophan, therefore showing that the tryptophan prototrophy was successfully conferred by the bait plasmid pAS1. The next set of plates contained media lacking tryptophan and histidine, both in the absence (plate c) and in the presence (plate d) of the chemical inhibitor 3-aminotriazole (3-AT). These plates were used to test for the selection marker gene *HIS3* activation in the absence of library plasmid. Y190 was unable to grow, however both the Y190 strains containing the PP2A catalytic subunits and the positive control SNF1/SNF4 showed growth. This shows the residual expression of the *HIS3* gene in Y190 which is diminished in the presence of the chemical inhibitor as seen by the reduced growth on plate d containing 3-AT. In the positive control SNF1/SNF4, reporter gene activation should occur and the strain should show increased growth compared to Y190 which only contains the bait plasmid. However, as there is no selective pressure on the yeast strain to maintain the plasmid containing SNF4, it is suspected that the interacting partner necessary for full *HIS3* activation has been lost in these colonies, thereby causing a similar growth pattern as seen with Y190/pAS1-C1 and Y190/pAS1-C3. Plates e and f were testing for activation of the reporter gene *lacZ*. All four strains were patched onto plates lacking tryptophan but containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). No blue color was seen on plate e indicating no reporter gene activation in the absence of a possible interaction partner. Plate f additionally lacked leucine, therefore selecting for the presence of the library plasmid pACT.

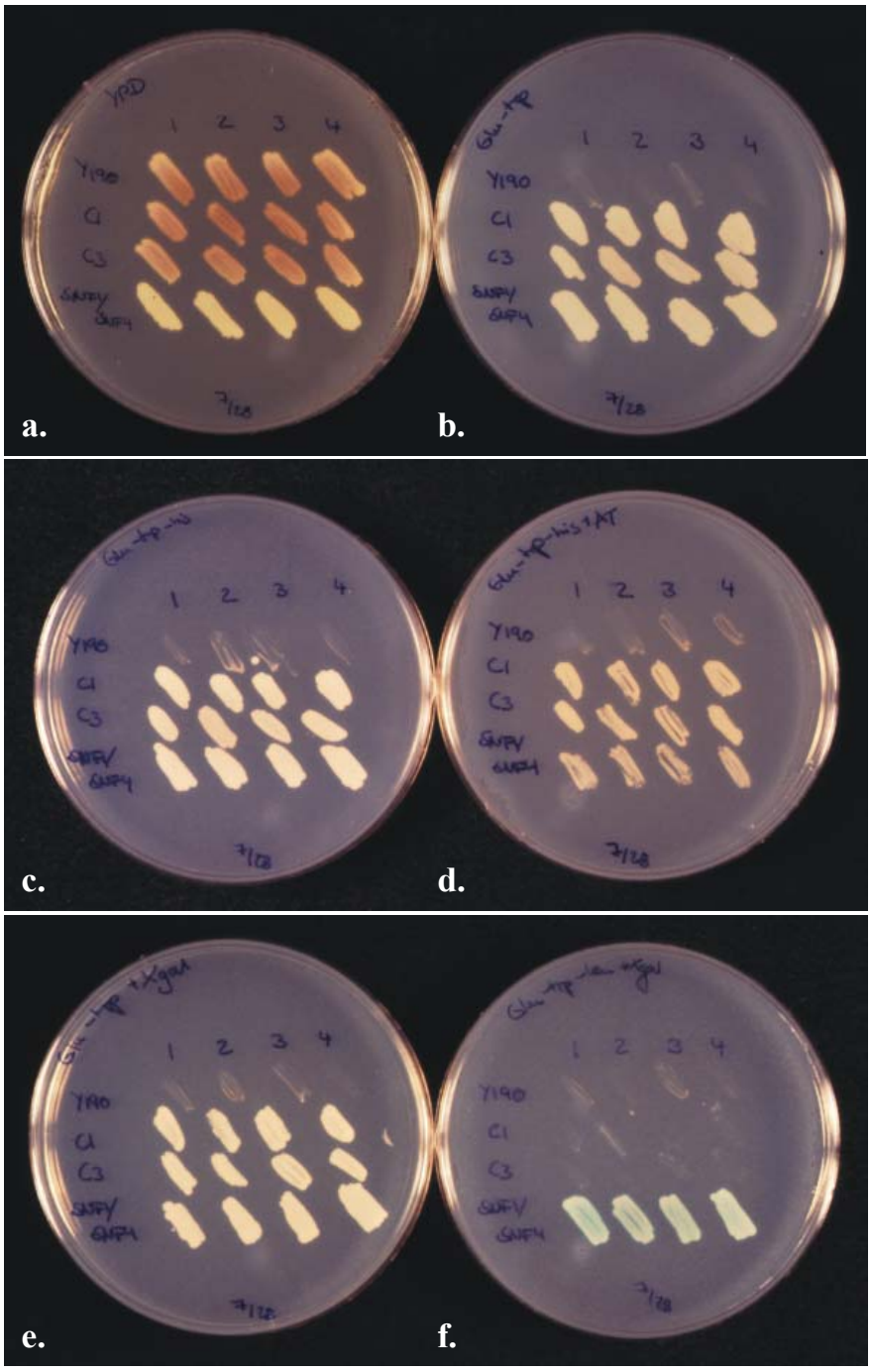

Figure 22: Control experiments for yeast two-hybrid screen

Four cell patches each of yeast Y190, Y190 transformed with pAS1 encoding the *A. thaliana* PP2A C-1 or C-3 subunit and Y190 containing a positive control for the two-hybrid screen were tested on different media plates (rows from top to bottom of each plate, respectively).

Plate a. Cells of all strains are viable of full yeast media. Plate b. Testing tryptophan prototrophy conferred by bait plasmid pAS1: Sustained growth on media lacking tryptophan. Plates c, d. Testing selection marker expression (*HIS3*): Showing residual *HIS3* expression even without interaction (plate c), however growth is greatly diminished in the presence of the chemical inhibitor 3-aminotriazole (3-AT, plate d). Plates e, f. Testing reporter gene expression (*lacZ*): No production of blue colonies indicating lack of reporter gene expression without the presence of an interactor for the bait protein (plate e). The positive control SNF1/SNF4 shows blue colonies when plated on media selecting for the library plasmid (plate f).

Y190 and Y190 only containing pAS1 with the cDNAs for the PP2A C-1 and C-3 subunits showed leucine auxotrophy as expected and were therefore unable to grow on this media. However, SNF1/SNF4 was able to sustain growth by maintaining the plasmid pACT encoding SNF4. The presence of both SNF1 and SNF4 enabled interaction and consequently it also showed reporter gene activation visible by the blue coloring of the yeast cells.

These controls showed the expected results for Y190 transformed with pAS1 containing the cDNAs for the *A. thaliana* PP2A C-1 or C-3 subunit. Both strains showed tryptophan prototrophy, but leucine auxotrophy. No reporter gene activation for *lacZ* was seen and the residual *HIS3* expression is greatly diminished in the presence of the chemical inhibitor to an even greater extent in single colonies than in cell patches (data not shown). Therefore, both Y190/pAS1-C1 and Y190/pAS1-C3 could be used as bait in the intended yeast two-hybrid screen.

4.2.4.4. Transformation of Plasmid Library into Yeast containing the C-1 and C-3 Bait

Upon successful completion of controls, transformations were performed to introduce plasmid DNA containing cDNA libraries into Y190/pAS1-C1 and Y190/pAS1-C3 to search for possible interacting proteins of the *A. thaliana* PP2A catalytic subunits C-1 and C-3.

The yeast strain Y190/pAS1-C1 was transformed with the cDNA library from adult *A. thaliana* plants, whereas Y190/pAS1-C3 was transformed with cDNAs isolated from seedlings. As shown in Tables 9 and 10, a total of 9 transformations (T 1 - 9) were performed. Transformations were performed using either the high efficiency transformation protocol according to Schiestl and Gietz, 1989 (T 1 - 8) or a protocol for large scale transformation modified from the Clontech Manual for MATCHMMAKER Two-Hybrid System (T 9).

For all transformations, a small volume of cells (1/20 to 1/750 of total volume of transformed cells) was plated on media lacking tryptophan and leucine. This selection for the presence of both the bait and the library plasmid was performed to determine the transformation efficiency.

The majority of the transformed cells were plated onto media lacking tryptophan, leucine and histidine and containing the chemical inhibitor 3-AT. This allowed for selection for both the bait plasmid, library plasmid as well as for *HIS3* selection marker activation simultaneously.

For transformations 1 - 4, colonies growing on these plates were picked and patched onto plates selecting for both bait and library plasmid. Cells were then replica plated onto media repeating the selection for *HIS3* gene activation as well as onto plates selecting for *lacZ* reporter gene activation.

Patches showing both growth and blue color indicated activation of both reporter gene systems and cells were picked from these patches. To rule out false positives due to various effects within a cell patch causing blue color, cells were streaked and blue color in single colonies was recorded.

Table 9: Screening results with *A. thaliana* PP2A catalytic subunit **C-1** as bait protein
Yeast Y190 containing pAS1-C1 was transformed with cDNA library CD4-10 constructed from mature leafs and roots of *A. thaliana*.

transformation (amount of plasmid DNA used in transformation)	total number of trans-formants	transformation efficiency (transformants/ µg DNA)	His^+	$lacZ^+$ (dark blue)	$lacZ^+$ (light blue)	blue in single colonies
C1-1 (0.63 µg DNA)	400	666	83	-	-	-
C1-2 (3.15 µg DNA)	1200	380	308	-	-	-
C1-3 (3.15 µg DNA)	600	190	304	-	-	-
C1-4 (3.15 µg DNA)	1000	317	272	-	1	-
C1-5 (4.41 µg DNA)	1040	236	509	4	16	2
C1-6 (4.41µg DNA)	480	109	128	-	-	-
C1-7 (4.41µg DNA)	500	113	201	-	-	-
C1-8 (4.41µg DNA)	520	118	295	7	65	2
	subtotal: 5740					subtotal: 4
C1-9 (18.9µg DNA)	23535	1245	8708	1	24	4
	total: 29275					total: 8

Table 10: Screening results with *A. thaliana* PP2A catalytic subunit **C-3** as bait protein
Yeast Y190 containing pAS1-C3 was transformed with cDNA library CD4-22 constructed from etiolated *A. thaliana* seedlings.

transformation (amount of plasmid DNA used in transformation)	total number of trans-formants	transformation efficiency (transformants/ µg DNA)	His^+	$lacZ^+$ (dark blue)	$lacZ^+$ (light blue)	blue in single colonies
C3-1 (0.59 µg DNA)	100	166	65	1	2	2
C3-2 (2.95 µg DNA)	2600	881	893	-	8	3
C3-3 (2.95 µg DNA)	4200	1424	1101	2	16	6
C3-4 (2.95 µg DNA)	7400	2508	772	1	10	5
C3-5 (4.13 µg DNA)	1820	441	704	-	40	1
C3-6 (4.13 µg DNA)	1900	460	956	3	37	1
C3-7 (4.13 µg DNA)	860	208	603	-	30	1
C3-8 (4.13 µg DNA)	420	102	232	-	41	5
	subtotal: 19300					subtotal: 24
C3-9 (17.7µg DNA)	15000	847	5220	1	61	4
	total: 34300					total: 28

For transformations 5 - 9, colonies growing after transformation were tested for *lacZ* activity using a filter lift assay. Cells showing blue color on the filters were then picked and grown on media selecting for both bait and library plasmid. These cells were then replica plated onto media repeating the selection for *HIS3* gene activation as well as confirming the *lacZ* reporter gene activation. Again, cells were streaked for single colonies and colonies showing blue color were recorded as positives.

A total of 6×10^4 transformants were screened and 34 % of those transformants showed reporter gene *HIS3* activation. A total of 36 positives also showed reporter gene *lacZ* activation indicating

an interaction between bait protein and library protein causing the activation of both reporter gene systems (as shown in Fig. 18). Of the 36 positives, eight were isolated in the screen using PP2A catalytic subunit C-1 (see Tab. 9) and 28 were isolated using the catalytic subunit C-3 (see Tab. 10) as bait protein. The catalytic subunit C-1 was used with a library made of mature leafs and roots, whereas C-3 was used with a library from 3-day old etiolated seedlings. The difference between C-1 and C-3 positives could be due to a difference in quality between the two libraries used in the screen. However, it might also be possible that this result indicates that PP2A is involved in more processes in the dark and development of seedlings than it is in processes within mature leafs and roots.

Due to the different representation of high-copy and low-copy mRNAs within a cDNA library, the recommended number of transformants is 5-10 times the number of genes within the genome to ensure saturation of the screen. With *Arabidopsis* having an estimated 25,000 genes encoded within its genome, a total number of 125,000 - 250,000 transformants should have been screened to reach saturation. In this study, 60,000 transformants were screened and 36 potential interacting partners for *Arabidopsis* PP2A catalytic subunits C-1 or C-3 were found. Therefore, it can be expected that the number of potential interacting proteins would have been even higher in a saturated screen.

The significance of the observed interactions needs to be determined with additional tests. The catalytic subunits used in this screen may show interactions with proteins that may not naturally occur within the cell. The PP2A enzyme is comprised of a core heterodimer build by the catalytic subunit and an A-regulatory subunit which is then completed to the holoenzyme by addition of another regulatory subunit from the B family. The holoenzyme is targeted to specific sub-cellular compartments, the substrate specificity is influenced and even the catalytic activity is modified by the regulatory subunits (Luan, 2003). During the screen, the catalytic subunits C-1 and C-3 may interact with proteins that *in vivo* might not be in the same cellular compartment as a holoenzyme containing one of these catalytic subunits. The *in vivo* yeast two-hybrid system is designed to be very sensitive, therefore interactions may show up that would for various reasons never occur. Theses false positives could be avoided through a more specific test for interaction like *in vitro* co-immunoprecipitation, however one would risk loosing the chance to pick up weak interactors. Even considering all the limitation of the performed two-hybrid screen, it still is an effective tool to get a first insight into processes in which dephosphorylation by PP2A could play an important role. Therefore, the isolated positives from the screening process were further examined.

4.2.4.5. Isolation of Library Plasmids encoding C-1 and C-3 Interaction Partners

For all positive yeast transformants, the library plasmid was isolated and sequenced in order to identify the potential interacting protein.

For this purpose, a loss of bait plasmid experiment was performed. Overnight cultures of the identified positives were grown in medium selecting only for library plasmid. Cells were then plated in low density onto the same medium. After colonies developed on the plate, the cells were replica plated onto medium selecting for both bait and library plasmid. Colonies that did not show growth when selecting for bait plasmid marker indicating the loss of bait plasmid were selected off the master plates and new overnight cultures were started.

Plasmid DNA was isolated from the yeast cells and used to transform *E. coli* JS5 cells. Plasmid DNA was then isolated from the *E. coli* cells and a restriction digest was performed to check for inserts in the library plasmids. The enzyme *Xho*I was used to release the library cDNA insert and all positives showed inserts of varying sizes ranging from 0.5 - 1.5 kb (data not shown). This met the size expectations for inserts as both libraries used for screening were constructed using cDNA in this size range.

The isolated plasmids containing cDNA inserts encoding for proteins possibly interacting with PP2A catalytic subunits C-1 and C-3 were then used for sequence analysis.

4.2.5. Results of the Two-Hybrid Screen

The sequences of the cDNA inserts contained in library plasmids showing activation of both reporter gene systems during the yeast two-hybrid screen were analyzed. Sequencing was performed both as described in Material and Methods and at the Yale Medical School DNA Sequencing Group by PCR based sequencing using an ABI Prism 377 Genetic Analyzer.

At the time of this study sequences were analyzed using the Wisconsin GCG package (version 9.1, 1997) and compared against the database sequences in the Stanford *Arabidopsis* Sequence database (http://genome-www.stanford.edu). Recently, all sequences were reformatted using the Sequence Utilities at BCM Search Launcher (http://www.searchlauncher.bcm.tmc.edu) and Blast searches were performed at the website of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST) and the Munich Information Center for Protein Sequences (http://mips.gsf.de). The results of the sequence analysis are detailed in Tab. 11.

Table 11: Summary of isolated interaction partners for both catalytic subunits C-1 and C-3 of *A. thaliana* PP2A
(*): Asterisk denotes matches made with recently available sequence data, unknown at time of work.

Interaction partners of PP2A catalytic subunit **C-1**

Isolated Positive	Obtained Sequence Data	MIPS code	Description (length and percentage of homology)
C1-1	769 bp	At3g15950	(*) unknown protein, similar to DNA topoisomerase IV subunit A (637 bp / 96%)
C1-2	774 bp	At3g15950	same as C1-1 (635 bp / 96%)
C1-3	762 bp	At1g13690	AtE1, stimulates ATPase activity of DnaK/DnaJ (351 bp / 99%)
C1-4	766 bp	At3g45640	AtMPK3, mitogen-activated protein kinase (495 bp / 93%, 145 bp / 91%)
C1-5	775 bp	At5g26280	(*) unknown protein, MATH domain containing protein (similar to C3-25) (312 bp / 95%)
C1-6	782 bp	At4g16390	(*) salt-inducible protein, nearly identical to chloroplastic RNA-binding protein P67 (704 bp / 92%)
C1-7	774 bp	At4g21560	(*) unknown protein, similar to vacuolar protein sorting-associated protein family (VPS28) (491 bp / 99%)
C1-8	764 bp	At3g26520	tonoplast intrinsic protein (AtTIP1;2), salt-stress inducible TIP (715 bp / 97%)

Interaction partners of PP2A catalytic subunit **C-3**

Isolated Positive	Obtained Sequence Data	MIPS code	Description (length and percentage of homology)
C3-1	no sequence data	-	-

Interaction partners of PP2A catalytic subunit **C-3** (cont'd.)

Isolated Positive	Obtained Sequence Data	MIPS code	Description (length and percentage of homology)
C3-2	174 bp	At2g43760	putative molybdopterin synthase, large subunit (67 bp / 86%)
C3-3	597 bp	At2g30870	glutathione *S*-transferase (erd13, *At*GSTF10) (537 bp / 99%)
C3-4	151 bp	At4g09650	(*) ATPase delta subunit (AtpD) (147 bp / 91%)
C3-5	247 bp	At5g10450	14-3-3-like protein AFT1 (250 bp / 94%)
C3-6	252 bp	At1g78040	(*) phosphoglycerate mutase 1 like protein (252 bp / 97%)
C3-7	218 bp	At2g47400	putative chloroplast protein CP12 (188 bp / 96%)
C3-8	607 bp	At1g60710 and At1g60730	auxin-induced bZIP transcription factor gene (ATB2), and similar to auxin-induced atb2 (237 bp / 99%, 205 bp / 99% and 236 bp / 90%)
C3-9	113 bp	At1g03600	unknown protein (112 bp / 97%)
C3-10	599 bp	At2g32150	(*) haloacid dehalogenase-like hydrolase family protein (574 bp / 100%)
C3-11	205 bp	At5g10360	40S ribosomal protein S6 (73 bp / 95%, 67 bp / 97%, 51 bp / 100%)
C3-12	191 bp	-	(*) sequence on chromosome 5 (189 bp / 92%)
C3-13	187 bp	At4g05050	polyubiquitin (UBQ11) (172 bp / 100%)
C3-14	183 bp	AT4G08950	(*) putative phi-1-like phosphate-induced protein (184 bp / 99%)
C3-15	185 bp	At1g60950	ferredoxin precursor (186 bp / 95%)

Interaction partners of PP2A catalytic subunit **C-3** (cont'd.)

Isolated Positive	Obtained Sequence Data	MIPS code	Description (length and percentage of homology)
C3-16	175 bp	At2g18960	plasma membrane proton ATPase (PMA) (157 bp / 96%, 48 bp / 85%)
C3-17	605 bp	At5g52060	(*) putative protein containing BAG domain (602 bp / 99%)
C3-18	599 bp	At3g25070	(*) RPM1-interacting protein 4 (RIN4) (447 bp / 96%, 142 bp / 97%)
C3-19	611 bp	At5g14840	(*) putative protein, similar to pyrroline-5-carboxylate reductase (598 bp / 99%)
C3-20	600 bp	At1g59870	(*) putative ABC transporter (591 bp / 99%)
C3-21	606 bp	At2g44670	(*) unknown protein, similar to senescence-associated protein SAG102 (448 bp / 99%, 164 bp / 100%)
C3-22	755 bp	At2g18110	(*) putative elongation factor 1-beta (224 bp / 89%, 375 bp / 87%)
C3-23	758 bp	At5g56100	(*) unknown protein, contains oleosin domain (627 bp / 98%)
C3-24	742 bp	-	(*) cloning vector pACT2 (Clontech) (628 bp / 94%)
C3-25	753 bp	At5g26260	(*) unknown protein, MATH domain containing protein (similar to C1-5) (624 bp / 98%)
C3-26	794 bp	At4g33220	pectinesterase like protein (694 bp / 88%)
C3-27	753 bp	At1g20620	unknown protein, identical to catalase 3 (SEN2) mRNA (491 bp / 98%)
C3-28	790 bp	At2g15570	putative thioredoxin M (571 bp / 94%, 143 bp / 100%)

In summary, a total of 32 potential interacting proteins were identified. No sequence information was obtained from one of the isolated library plasmids, whereas within another plasmid only sequence information matching the library plasmid itself was determined. Twenty-two of the

isolated positives were identified as known proteins and 10 were classified as unknown or putative proteins with varying degrees of similarity to proteins with known functions. One of the proteins was isolated twice (C1-1 and C1-2) and another pair of positives were very similar to each other (C1-5 and C3-25). Due to the fact that the libraries used were prepared from cDNAs, it was surprising that one of the library plasmids carried *Arabidopsis* DNA that had neither MIPS code nor open reading frame for a putative protein assigned (C3-12).

4.2.5.1. Confirmation of C-1 and C-3 Interaction Specificity

For some of the isolated potential interaction partners, further experiments were performed to confirm the interaction in a 1:1 transformation as well as test the interaction with both *Arabidopsis* PP2A catalytic subunits.

To show that the library plasmid was indeed responsible for the observed interaction causing the activation of both reporter gene systems, the isolated library plasmid was used to re-transform the yeast strain expressing the potentially interacting PP2A catalytic subunit. The isolated positives C3-5, C3-13, C3-15 and C3-16 were used to transform the yeast strain Y190/pAS-C3. The cells were plated onto media selecting for both the bait and library plasmid and then replica plated to test for reporter gene activation. All four positives showed growth on medium lacking histidine while containing the chemical inhibitor 3-AT and blue color on medium containing X-gal, thereby confirming the interaction of the library protein with the bait PP2A catalytic subunit C-3 (data not shown).

In order to test a possible interaction with other catalytic subunits, isolated library plasmids were used to transform the yeast strain containing the other PP2A catalytic subunit. The plasmid DNA samples of C3-5, C3-13, C3-15 and C3-16 were also used to transform the yeast strain Y190/pAS-C1. The same procedure was followed to test for reporter gene activation and all four showed the same results as with C-3 (data not shown). This indicated the same interaction of the library protein with both the *A. thaliana* PP2A catalytic subunits C-1 and C-3. This result was not unexpected due to the high sequence similarity between the catalytic subunits C-1 and C-3. The specificity for interactions *in vivo* is expected to be conferred by the regulatory A- and B-subunits. However, for the purpose of this screen, both catalytic subunits were used without regulatory subunits present.

4.2.5.2. Characteristics of Potential Interaction Partners from the Two-Hybrid Screen

The identified potential interaction partners from the two-hybrid screen using the *A. thaliana* PP2A catalytic subunits C-1 and C-3 can be divided into 3 major groups.

In the first group are unknown proteins with no further information about their biological functions. For some of these positives, sequence analysis has revealed putative protein domains which could give an indication about potential roles. It is unclear at this point why an interaction with protein phosphatase 2A might occur. However, the fact that these proteins were identified as possible interaction partners of PP2A might help elucidate their functions in the future.

The second group is comprised of known proteins for which the reason for the proposed interaction with PP2A is unclear. For these proteins, further research will either show evidence of the connection to protein phosphatase 2A and the need for dephosphorylation or will prove that the observed interaction in the two-hybrid screen was unspecific.

The third group represents proteins where the interaction with PP2A is conceivable and, in some cases, has since been shown in other studies.

Following is a more detailed description of some of the isolated proteins in an attempt to determine the plausibility of the potential interaction.

Unknown protein, similar to DNA topoisomerase IV subunit A (C1-1 and C1-2)

DNA topoisomerase IV is a topoisomerase type 2 enzyme capable of cleaving two DNA strands in an ATP-dependent reaction and passing DNA through the double-strand break. The enzyme was originally identified in prokaryotes and was shown to be responsible for unknotting DNA in *E. coli* (Deibler *et al*., 2001), a process necessary to avoid possible double-strand breaks within the DNA molecules. The enzyme structure is a dimer composed of two subunits.

Although no possible dephosphorylation of the A subunit has been described in the literature reviewed, some credibility is given by the fact that this protein was the only one identified twice as potential interactor.

AtE1 (C1-3)

AtE1 was originally described as potential cytoplasmic GrpE homologue and was said to stimulate the ATPase activity of DnaK/DnaJ (Miernyk and Kroczynska, EMBL submission, 1996). DnaK (Hsp70) in *E. coli* is involved in protein folding and translocation across membranes in an ATP-dependent manner. This molecular chaperone function is enhanced by the association with the chaperone DnaJ and the nucleotide exchanges factor GrpE which catalyzes peptide release from the chaperone complex. Dionisi *et al*., 1998, could show that the Hsp70 chaperone system

(including DnaK, DnaJ and GrpE) is required for the productive folding of pea chloroplast ferredoxin-$NADP^+$ reductase (FNR) in *E. coli*. This represents a possible connection to the positive C3-15, which identified as a ferredoxin precursor.

Recently performed sequence comparisons of AtE1 revealed a high similarity to peptidyl-prolyl cis-trans isomerase, which describes the activity of cyclophilins, a group of proteins that are highly conserved among species. However, AtE1 is not mentioned as member of the *Arabidopsis* cyclophilin gene family (Romano *et al*., 2004). Cyclophilins are ubiquitous proteins that are involved in a variety of processes including protein folding, protein transport across cellular membrane, heat shock response, receptor complex stabilization, apoptosis, receptor signaling, RNA processing and splicosome assembly. In *A. thaliana,* an interaction of the cyclophilin ROC7 with a regulatory PP2A A-subunit (*rcn1*) has been shown (Jackson and Söll, 1999). The interaction was found through a two-hybrid screen and confirmed by mutational analysis within the yeast system. Further analysis indicated that RCN1 and ROC7 function together in at least one signal transduction pathways regulation root growth in *Arabidopsis*.

Both possible functions of AtE1 indicate different plausible interactions with protein phosphatase. However, the protein would have to be analyzed further to determine its biological purpose first.

AtMPK3, mitogen-activated protein kinase (MAP kinase, C1-4)

As PP2A has been shown to influence MAP kinases in mammalian systems (Quintaje *et al*., 1996), a very likely interaction partner for protein phosphatase 2A was found with AtMPK3. This mitogen-activated protein (MAP) kinase is a serine/threonine kinase and was described to be part of an oxidative stress-activated MAPK cascade in plants. This cascade was shown to activate stress-responsive promoters that react to heat shock, cold and drought and to repress auxin-inducible promoters (Kovtun *et al.*, 2000). Additionally, AtMPK3 has been described as a H_2O_2-activated mitogen-activated kinase that is involved in down-regulating the accumulation of reactive oxygen species and thereby enhancing the tolerance of *Arabidopsis* plants to multiple abiotic stresses (Moon *et al*., 2003). Asai et al., 2002, could identify the MAP kinase 3 as part of a signaling pathway conferring resistance to both bacterial and fungal pathogens in *Arabidopsis*. The same kinase also shows increased transcript levels as reaction to mechanical stimuli, low temperature and osmotic stress and is co-induced with ATPK19, a S6 ribosomal protein kinase (Mizoguchi *et al.*, 1996) which could imply a connection to the isolated protein C3-11.

Salt-inducible protein, nearly identical to chloroplastic RNA-binding protein P67 (C1-6)

P67 is a 67 kDa protein that is addressed to the chloroplast. It shows a highly specific RNA-binding activity and *in vitro* only recognizes a pre-rRNA fragment. The natural substrate in the chloroplasts still needs to be determined, but the most likely function of the protein is in chloroplastic RNA processing (Lahmy *et al*., 2000). Due to its location in the chloroplast, the potential interaction with PP2A could only occur if a regulatory subunit confers this subcellular location to the PP2A holoenzyme. The isolated protein appears to be salt-inducible and therefore an interaction would imply a salt-stress mediated PP2A response that reaches into the chloroplast.

Vacuolar protein sorting-associated protein VPS28 (C1-7)

In yeast, vps28 was isolated as a vacuolar protein sorting mutant. It was determined to be a cytoplasmic protein and disruption of *VPS28* results in moderate defects in both biosynthetic traffic and endocytic traffic destined for the vacuole. Possibly as coat protein, Vps28p may facilitate the formation of transport intermediates required for efficient transport out of prevacuolar endosome functioning as intermediate compartment in the transport between Golgi and vacuole (Rieder *et al.*, 1996). A possible interaction with protein phosphatase 2A would imply a role for dephosphorylation in the regulation of various transport processes (see C3-20) including protein transport.

Tonoplast intrinsic protein AtTIP1;2 (C1-8)

AtTIP1;2 was isolated as a salt-stress inducible protein and is part of a family of major intrinsic proteins (MIPs) that facilitate the passive transport of small polar molecules like water and/or glycerol across membranes. Within this family, proteins that specifically transport water are called aquaporins (AQP). Tonoplast intrinsic proteins (TIPs) build a subfamily, are expressed in subcellular compartments of the tonoplast and have AQP activities. The permeability of membranes is regulated by the number of MIPs present, but also in some cases by the phosphorylation/dephosphorylation status of the channels (Johanson *et al.*, 2001).

Azad *et al.*, 2004, could show that a water channel protein AQP in tulips, which is located in plasma membrane, was phosphorylated at Ser residues. The putative plasma membrane aquaporin functions as a substrate for purified PP2A catalytic subunit and holoenzyme from tulip *in vitro*. Therefore, a potential interaction of *Arabidopsis* PP2A with AtTIP1;2 seems plausible and implies a role for protein phosphatase 2A in the response to salt stress.

Glutathione *S*-transferase erd13 (C3-3)

ERD13 was isolated as dehydration-inducible gene (Kiyosue *et al.*, 1993) and was shown to encode a glutathione *S*-transferase (GST). GSTs are abundant proteins encoded by a super-family of genes with 47 members in *A. thaliana*. GSTs form dimers which bind glutathione and hydrophobic ligands, thereby attaching glutathione to for example electrophilic xenobiotics like herbicides. This tags the *S*-glutathionylated metabolites for vacuolar import by ATP binding cassette (ABC) transporters (see C3-20) and leads to vacuolar sequestration. The glutathione *S*-transferases play a crucial role in the detoxification of both endogenous and xenobiotic compounds. Additional functions in the plant metabolism are still unclear, but individual GSTs show different responses to many forms of biotic and abiotic stress (Edwards *et al.*, 2000; Wagner *et al.*, 2002). Rao and Clayton, 2002, showed that human PP2A activity can be regulated by glutathionylation. Given these results, it seems possible that *Arabidopsis* PP2A either plays a role in the detoxification process or is itself target of regulation by glutathione.

14-3-3 like protein AFT1 (C3-5)

14-3-3 proteins are ubiquitous eukaryotic phosphoserine/phosphothreonine-binding proteins and are encoded by a gene family of at least 12 members in *A. thaliana*. Most known targets of 14-3-3 proteins in animals are involved in signal transduction and transcription, whereas in plants the target proteins are involved in metabolic regulation. The inhibition of nitrate reductase and other enzymes of carbon and nitrogen metabolism have been described suggesting a function as a global control mechanism in response to changing nutrient levels. Additionally, 14-3-3 proteins are implicated in earlier points of the same signaling pathways in carbon and nitrogen metabolism by interacting with different kinases and transcription factors. Roles in regulating plant development have been suggested as well as the involvement in the transcriptional response of pathogen defense. Fungus resistance has been shown to be caused by activation of a plasma membrane H^+-ATPase (see C3-16) through the interaction with a 14-3-3 protein. Members of this gene family are also involved in the response to abiotic stresses such as low temperature and osmotic stress, nuclear-cytoplasmic shuttling and protein import into organelles. 14-3-3 proteins function by interacting with target proteins and providing a scaffold. While interacting with two proteins, this results in bringing those proteins together. Conversely, 14-3-3 protein can prevent the interaction of two proteins by binding to one, but not to the other. Intramolecular scaffolding can induce conformational change or bring functional domains into juxtaposition. 14-3-3 protein activity itself is regulated by phosphorylation and in animal systems, both an interaction with PP1 and PP2A has been described (reviewed by Roberts, 2003; Dougherty and Morrison, 2004).

AFT1 has been described to activate transcription in yeast. The protein was used as bait in a yeast two-hybrid screen fused to the bacterial LexA binding domain and was able to activate reporter gene transcription without interacting with the transcription activator domain. However, the presence of the LexA binding domain was necessary for this activation. Even though *in vivo* functions of ATF1 are still unclear, a possible function as transcription activator was concluded (Wang *et al.,* 1999).

During this study, ATF1 was isolated as potential interaction partner of PP2A while being fused to the activation domain. Therefore, even though ATF1 might be able to activate transcription by itself, it seems that the interaction to a catalytic subunit of PP2A is necessary to get in close contact of the binding domain within the yeast two-hybrid system. Considering the suggestions of 14-3-3 regulation by phosphorylation, the interaction of ATF1 and protein phosphatase 2A in *Arabidopsis* is plausible.

Phosphoglycerate mutase (C3-6)

This isolated potential interaction partner shows a similarity to a group of cofactor-dependent phosphoglycerate mutase/biphosphoglycerate mutase enzymes. They are key catalysts of glycolysis that have been well characterized in animals, but not plants. A homologue belonging into this group was isolated from *Arabidopsis* and shown to be up-regulated upon infection with nematodes (Mazarei *et al*., 2003). The apparent role of this enzyme in plant-pathogen interaction implies a need for regulation and the interaction with protein phosphatase 2A could provide a mechanism for this regulation.

CP12 (C3-7)

CP12 is a small nuclear encoded chloroplast protein of higher plants which interacts with key enzymes of the reductive pentosephosphate cycle (Calvin cycle). Wedel *et al.*, 1997, concluded that the described light regulation of the Calvin cycle functions not only through the reductive activation by the ferredoxin/thioredoxin system, but also through the interaction and reversible dissociation of key enzymes with CP12 as linker in a supramolecular complex. (Graciet *et al*., 2003). Like the isolated P67 (C1-6), CP12 is also a protein located in the chloroplast. Therefore, a possible interaction of protein phosphatase 2A with CP12 and a regulatory role in the Calvin cycle would depend on the subcellular location of PP2A in the chloroplast.

Auxin-induced bZIP transcription factor gene ATB2 (C3-8)

ATB2 is a transcription factor that is expressed in a light-induced and tissue-specific way. The activity of the transcription factor is repressed by sucrose and this repression is post-transcriptional, most likely through translational control. ATB2 has a complex leader sequence. Mechanisms for translation control often involve the interaction of leader sequences with mRNA binding proteins. The carbohydrate-mediated inhibition of this light-induced gene expression shows the close relationship between light and carbohydrate signals in control of gene expression (Rook *et al*., 1998). No possible phosphorylation sites have so far been described for ATB2, however phosphorylation has been implicated as regulatory mechanism in several auxin responses, at the junction of light and auxin signaling pathways as well as for the activity of auxin-responsive transcription factors (DeLong *et al*., 2002). Therefore, a potential interaction of PP2A with ATB2 seems feasible.

Ribosomal protein S6 (C3-11)

The ribosomal protein S6 (RPS6) is located in the mRNA binding site of the 40S subunit of cytosolic ribosomes, a region involved in the initiation of translation. RPS6 has conserved phosphorylation sites and is considered the major phosphoprotein in eukaryotic ribosomes. RPS6 phosphorylation is thought to promote the selective translation of a subset of transcripts. The phosphorylation status of ribosomal protein S6 is modulated by different protein kinases and phosphatases that are activated in response to various external or internal signals. The protein's cognate kinase is S6 kinase, AtS6k which was shown to be involved in cell cycle progression in mammalian systems. A role for *Arabidopsis* S6 kinases has been described in MAP kinase pathways (see C1-4) responsive to cold, salt and drought stress and AtS6k has been shown to be activated by the phytohormones auxin and kinetin. Both protein phosphatase 1 and 2A have been implicated as negative regulators of RPS6 phosphorylation in animal and mouse systems. In *Arabidopsis* dephosphorylation of RPS6 is inhibited by okadaic acid, a known inhibitor of PP1/PP2A (described in Turck *et al*., 2004; Williams *et al*., 2003).

Therefore, an interaction of RPS6 with a catalytic subunit of PP2A detected during this study's two-hybrid screen serves to confirm the role of protein phosphatase 2A in the dephosphorylation of ribosomal protein S6.

Polyubiquitin UBQ11 (C3-13)

Ubiquitin is involved in the selective degradation of intracellular proteins through covalent ligation to proteins destined for degradation. Functional ubiquitin is produced by two types of

genes: ubiquitin extension genes and polyubiquitin genes. In *A. thaliana*, ecotype Columbia, five polyubiquitin genes in two subgroups, have been described and the mRNA expression of all genes is modulated independently. A diverse group of substrates are targeted through the ubiquitin pathway. In animals, the pathway responds to biotic and abiotic stresses, is involved in DNA repair, gene regulation, cell cycle regulation, signal transduction and cell recognition (Sun and Callis, 1997; Sun *et al*., 1997). Protein degradation through the ubiquitin-proteasome pathway starts with the covalent attachment of ubiquitin to a potential substrate by the sequential action of three enzymes with E3 being the specificity component of pathway (E1 - ubiqitin-activating, E2 - ubiquitin-conjugating, E3 - ubiquitin-protein-ligase). Polyubiquinated proteins are then recognized and degraded by the 26S proteasome. About 1200 (= 5%) of all predicted 25,000 proteins in *Arabidopsis* are estimated to function in ubiquitin-proteasome pathways. One of the possible processes identified in plants and showing an involvement of the ubiquitin pathway, is the phytochrome mediated repression of light-regulated development in the dark. COP1 is involved in HY5 degradation in dark and has a proposed E3 function together with additional interacting proteins in this process (Hellmann and Estelle, 2002). Also, protein degradation through the ubiquitin-proteasome pathway was recently implicated in plant defense against pathogens (reviewed in Devoto *et al*., 2003).

The possible interaction of protein phosphatase 2A with a polyubiquitin gene, opens up a function for phosphorylation in the regulation of targeted protein degradation through the ubiquitin-proteasome pathway.

Ferredoxin (C3-15)

Ferredoxin is probably encoded by two genes in *A. thaliana* and the proteins are involved in a variety of redox reactions in plants (photosynthesis, regulation of carbon metabolism, assimilation of nitrogen and sulfur compounds). Ferredoxins are found both in photosynthetic and non-photosynthetic tissue and have been shown to be light-regulated under phytochrome control in pea (Somers *et al.*, 1990).

A two-hybrid screen performed using a regulatory A-subunit of *Arabidopsis* PP2A (*RCN1*) also revealed a ferredoxin as interaction partner of PP2A (Ph.D. thesis of Karin Johnson, 1999).

The reversible electron transfer between ferredoxin and NADP(H) is catalyzed by ferredoxin-NADP(H) reductases (FNRs) (Ceccarelli *et al*., 2004). These FNRs have been suggested as possible interaction partner of AtE1 (two-hybrid screen positive C1-3), thereby increasing the possibility of a role for protein phosphatase 2A in the proper assembly of redox-based systems for the metabolism in plants.

Plasma membrane H^+-ATPase PMA (C3-16), ATPase delta subunit AtpD (C3-4)

In *A. thaliana,* plasma membrane H^+-ATPases are encoded by a multigene family with 11 members (AHA). The proteins are the major ion pumps in the plasma membrane of higher plants, in contrast to the animal system where Na^+/K^+-ATPases play the dominant role. The functional unit is a monomer and is organized as dimer or oligomer within the membrane which is different from other proton pumps including the vacuolar membrane H^+-ATPases. By generating an electrochemical proton gradient across plasma membrane, plasma membrane H^+-ATPases provide energy for proton-coupled transporters that are responsible for various transport processes. These include nutrient uptake, maintenance of cell turgor, control of water fluxes in stomatal guard cells and pulvinar motor cells that allow the movement of leaves in various plants. Additionally, the opening of stomata through accumulation of K^+ in guard cells is driven by an electrical potential across membrane that is created by an H^+-ATPase. The fungal toxin fusicoccin irreversibly activates plasma membrane H^+-ATPases causing stomatal opening resulting in wilting of leaves which will cause the eventual death of the plant. The activity of the pump responds to a variety of environmental conditions including salt stress, hormones (auxin) as well as light and pathogens. The H^+-ATPase activity in guard cells is physiologically regulated by blue light which gets absorbed by PHOT1 and PHOT2. These photoreceptors were shown to have kinase activity and cause phosphorylation, most likely indirectly, of the H^+-ATPase. The C-terminus of H^+-ATPase acts as autoinhibitory domain, has multiple phosphorylation sites and Ser/Thr phosphorylation/dephosphorylation has been shown to affect activity of pump. 14-3-3 proteins (see C3-5) bind to phosphorylated Thr residues within the H^+-ATPase, displaces the C-terminus of the protein and thereby leading to the activation of the enzyme The fungal toxin fusicoccin stabilizes the complex formation of 14-3-3 protein and H^+-ATPase even in absence of an phosphorylated Thr residue (Harper *et al*., 1990; recently reviewed in Sondergaard *et al*., 2004)
Due to the fact, that the activity of the plasma membrane H^+-ATPase is shown to be regulated by Ser/Thr phosphorylation makes the proton pump a very likely target for dephosphorylation by protein phosphatase 2A in *Arabidopsis*.

RPM1-interacting protein 4, RIN4 (C3-18)

RIN4 is involved in pathogen response of *Arabidopsis* against *Pseudomonoas syringae.* The protein RIN4 works as a negative regulator of basal defense, but is a positive regulator of an innate immune response. The protein is phosphorylated by effector proteins (virulence factors) from *Pseudomonoas syringae*. This targeting of RIN4 by effector proteins which cause post-transcriptional elimination of RIN4 is monitored and leads to rapid defense activation involving

resistance (R) genes. This leads to localized cell death around the site of infection and restriction of pathogen growth (Mackey *et al*., 2003).
Both C3-6 and C3-18 imply a role for *Arabidopsis* PP2A in plant-pathogen response mechanisms.

Pyrroline-5-carboxylate reductase (C3-19)

Pyrroline-5-carboxylate reductase (P5CR) is the last enzyme at the converging point of both proline biosynthetic pathways. The expression of the enzyme is differentially regulated during development and induced during salt stress. The accumulation of free proline is described as a response to osmotic and other stresses in many organisms and is thought to play a pivotal role in osmotic stress tolerance (Hua *et al*., 1997). This potential interaction partner is another indication for an involvement of PP2A in the response to salt stress in *A. thaliana*.

ABC transporter (C3-20)

ATP-binding cassette (ABC) transporter-like activities have originally been identified on vacuolar membrane of plant cells. The ABC transporter activities are directed toward large amphipathic organic anions and are directly energized by Mg-ATP instead of a transmembrane H^+-potential. Three different functions have been described including an activity competent in the transport of bile acids and the transports of chlorophyll catabolites resulting in excretion storage of linear tetrapyrroles from catabolism of chlorophyll during leaf senescence. Another function is as glutathione *S*-conjugate pump that transports glutathionated compounds into vacuolar sequestration (see C3-3). In *Arabidopsis*, a gene was isolated that encodes for a multi-specific ABC transporter competent in the transport of both glutathione conjugates as well as chlorophyll catabolites (Lu *et al*., 1998). A very recent report of an ABC transporter involved in export of wax precursor molecules (VLCFAs) from plant epidermal cells to form the epicuticular wax layer (waxy cuticle), also stated that the genomes of living land plants contain more than 100 ABC transporter genes (Pighin *et al*., 2004).
In mammalian cells, different members of the ABC transporter family transport a wide variety of substrates including hydrophilic molecules, drugs and lipids. Surviving cancer cells for example, express a multi-drug resistance (MDR) ABC transporter that pumps out anticancer drugs (Schulz and Frommer, 2004).
The fact that *Arabidopsis* PP2A showed a potential interaction with both a glutathione *S*-transferase and an ABC transporter, might imply a possible role for protein phosphatase 2A in a glutathionylation-dependent transport process.

Senescence-associated protein SAG102 (C3-21)

Leaf senescence is a highly regulated process that involves orderly, sequential changes in cellular physiology, biochemistry and gene expression leading to programmed cell death. A complex regulatory network involving many genes functions in the process reacting not only to endogenous developmental factors such as aging and hormones, but also to various environmental factors such as stress and nutrient supply. Several genes in different conceptual categories have been identified that are transcriptionally up-regulated in senescing leaves and they are collectively termed senescence-associated genes (SAGs) (Lim *et al*., 2003). The importance of a tight regulation in this process makes a possible involvement of dephosphorylation by PP2A plausible.

Elongation factor 1-beta (C3-22)

Translation is one of the many stages where regulation of gene expression can take place. The regulation may happen at one of the three stages of translation: initiation, elongation or termination. During elongation, aminoacyl-tRNAs enter the ribosome in a process mediated by eEF-1 which requires the hydrolysis of GTP. eEF-1 exists as heterotetramer made up of subunits α, β, γ and δ. The regulation of the eEF-1 complex activity was described to occur at the translation and post-translational level (Gidekel *et al*., 1996). A potential phosphorylation and dephosphorylation by PP2A mechanism could be involved in the regulation of this elongation factor and thereby defining a role for PP2A in protein synthesis regulation.

Pectinesterase (C3-26)

Pectin is one of the main components of the plant cell wall. It is secreted into the wall in a highly methylesterified form and subsequently de-esterified by pectin methylesterase (pectinesterase). The de-esterification is a prerequisite for pectin degradation by either plant or microbial enzymes. The cell wall modification plays an important role in the outcome of host-pathogen interactions. Variations in pectin compositions have been associated with resistance against several pathogens (Raiola *et al*., 2004; Vorwerk *et al*., 2004). An interaction of pectinesterase with protein phosphatase 2A would imply an even stronger role of PP2A in various aspects of plant-pathogen response.

Catalase 3 (C3-27)

The function of catalases represents the primary enzymatic mechanism in aerobic organisms to remove toxic H_2O_2 through its dismutation into water and oxygen. In *Arabidopsis*, a family of at least 6 isoenzymes was described which show distinct patterns of spatial expression. The enzyme is a heme-containing tetramer which is abundant in glyoxysomes in oil seed plants like

Arabidopsis and in leaf peroxisomes involved in photorespiration. Additional roles have been shown in the response to chilling stress and pathogen defense with an oxidative burst as earliest response during the non host resistance pathogen defense. Catalase 3 (CAT3) expression is regulated by the circadian clock, peaks in the evening and responds to a temperature sensitive mechanism (Zhong and McClung, 1996; Michael *et al*., 2003).
No involvement of phosphorylation has been described in the literature reviewed, but a potential interaction with protein phosphatase 2A could imply a role for PP2A in oxidative stress response in general and more specifically again in plant pathogen response.

Thioredoxin M (C3-28)

Thioredoxins (TRX) represent one of major protein disulfide reducing systems and play a role in the redox regulation of various processes. They are small ubiquitous proteins with a well conserved active site and are encoded by more than 20 genes in the *Arabidopsis* genome. TRXs are involved in the regulation of various processes such as the photosynthesis, Calvin cycle, regulation of transcription factors and the response to oxidative stress. Additionally, the folding of proteins and amino acid synthesis, metabolism, plant defense against herbivores and/or pathogens are influenced by thioredoxins. In the chloroplasts, two TRX isoforms have been identified with one target of TRX-M being a chloroplast cyclophilin (Marchand et al., 2004; Motohashi *et al*., 2003). This potential interaction partner again will require a subcellular location of PP2A into the chloroplast to be possible. However, other isolated potential interacting proteins have already implied a role for protein phosphatase 2A in protein folding which makes this interaction feasible.

4.2.5.3. Summary of Two-Hybrid Screen

The various isolated potential interaction partners indicate an involvement of protein phosphatase 2A in a wide range of cellular processes. Possible roles are in DNA replication, redox reactions, general stress response including dehydration. Several different isolated positives indicate an involvement in various aspects of plant-pathogen interaction. Additionally, osmoregulation, transport processes located at the vacuolar and plasma membrane as well as ubiquitin-dependent protein degradation are processes possibly influenced by PP2A. In conclusion, these results points towards a role of protein phosphatase 2A as a central molecular switch coordinating the adaption of *Arabidopsis* to important intra- and extracellular stimuli (see Fig. 23).
As described in Uetz *et al.*, 2000, large scale two-hybrid screens were used to identify protein-protein interactions between gene products from open reading frames provided from the *Saccharomyces cerevisiae* genomic sequence and their data revealed interactions that placed

proteins of so far unknown function into biological contexts. However, within this network no interactions of PP2A other than between catalytic and regulatory subunits were found. During this screen performed as part of this study none of the described regulatory A- or B-subunits have been identified. This matches results from two-hybrid screens performed using regulatory A-subunits from either *Arabidopsis* or humans as bait. Both screens failed to reveal catalytic or other regulatory subunits as potential interactors (Jackson and Söll, 1999; McCright and Virshup, 1995). To further confirm to observed interactions, more specific and more stringent experiments like *in vitro* co-immunoprecipitation assays would have to be performed. The potential interaction partners would have to be isolated and studied further to determine PP2A binding and dephosphorylation sites. This could then allow the elucidation of the exact role of protein phosphatase 2 A in different signal transduction cascades.

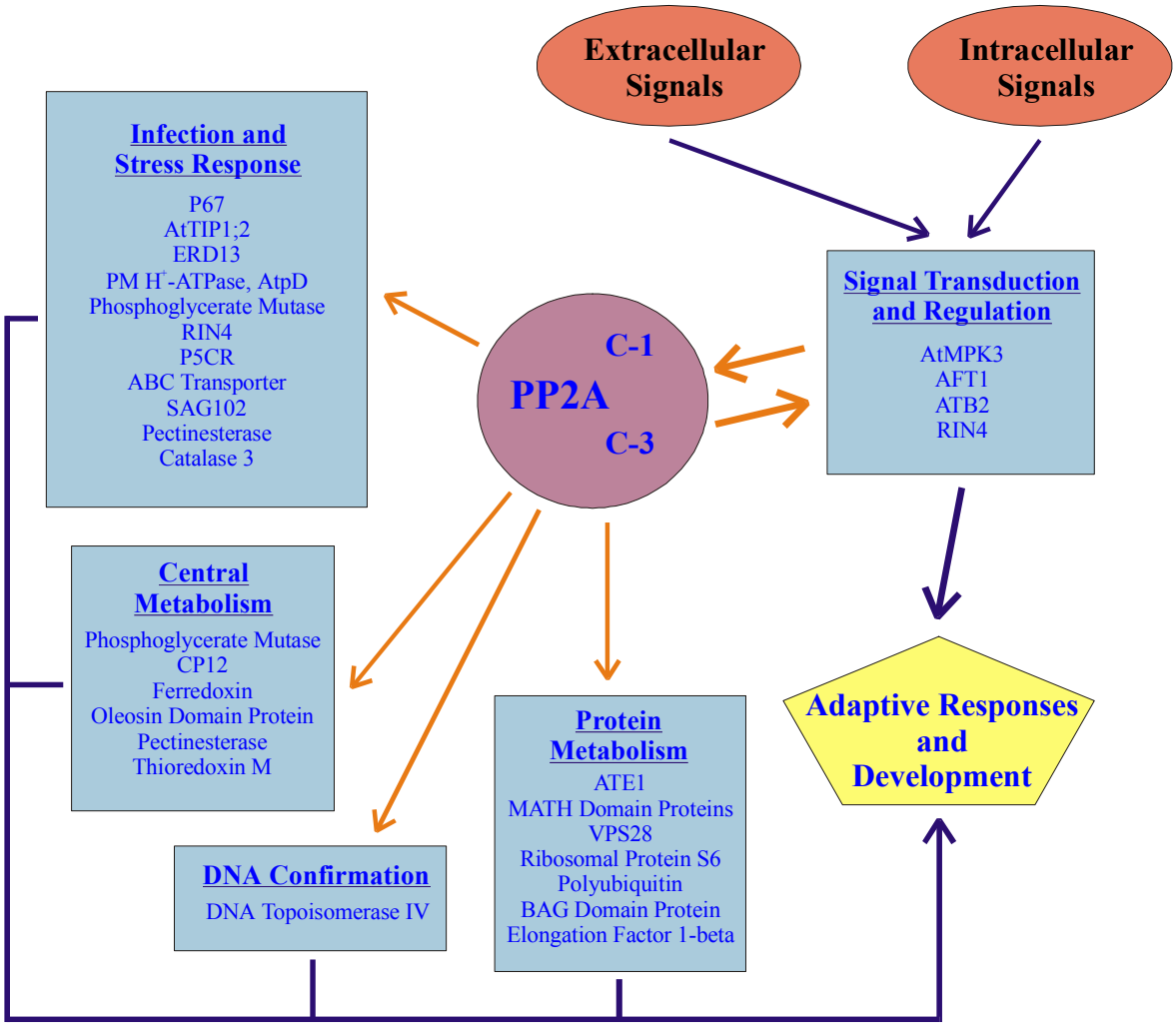

Figure 23: Model of protein phosphatase 2A as central molecular switch in *A. thaliana*
Schematic representation of potential interacting proteins isolated in the yeast two-hybrid screen using the PP2A catalytic subunits C-1 and C-3 as bait.

4.3. Screening of *Arabidopsis thaliana* AMAZE Collection for PP2A Subunit Mutants

Functions of protein phosphatase 2A (PP2A) in higher plants have been described in carbon and nitrogen metabolism, ion channel regulation, gene expression and control of cytoskeletal organization (recently reviewed in Luan, 2003). The isolation of the regulatory A subunit mutant *rcn1* of PP2A in *Arabidopsis* has defined a role in plant developmental processes like differential root elongation and auxin transport (Garbers *et al*., 1996).

As a different approach to obtain insight into signal transduction pathways involving protein phosphatase 2A, a PCR-based mutant screen was performed. It was attempted to identify *Arabidopsis* plants with a mutation within one catalytic subunit of protein phosphatase 2A. By observing possible phenotypes an indication of PP2A involvement in a particular process in *A. thaliana* could be obtained.

4.3.1. Description of the Mutant Collection

The screening for mutations within catalytic subunits of PP2A was performed at the Max-Planck-Institut für Züchtungsforschung in Köln, Germany with friendly permission of Prof. Dr. Klaus Palme (currently at the University of Freiburg, Germany). The collection of mutants was called "A Mutagenesis System for *Arabidopsis* at the Max-Planck-Institut für Züchtungsforschung using *En-1* Elements "(AMAZE).

The autonomous transposable element *En-1* from maize (*En/Spm*) shows frequent germinal excision coupled with re-integration in the heterologous host *A. thaliana*. After excision of the *En-1* element a 3 bp footprint in the gene sequence through target site duplication remains behind. These features make the *En-1* element a suitable system for transposon tagging in *Arabidopsis* (Cardon *et al.*, 1993; Wisman *et al*., 1998).

The resources of AMAZE included a collection of *Arabidopsis* seed stocks and genomic DNA preparations from about 3000 lines carrying approximately 15000 insertions of the maize transposable elements *En-1*. Within these lines the insertion of the *En-1* elements into specific genes can be investigated by PCR screens using primers complimentary to the edges of the *En-1* element and primers complimentary to the target gene.

To minimize the number of PCR reactions necessary for screening a 3-dimensional pooling strategy was used. The population was grown in 85 trays with 35 plants each in a total of seven rows and five columns. The population was divided in sub-populations of 16-18 trays each and each were labeled by a color. Leaf material was harvested in pools of 3-trays, 1-tray and pools of

all rows and columns with each plant thereby being represented in 4 pools. Fig. 24 and Tab. 12 describe the pooling strategy within the AMAZE collection.

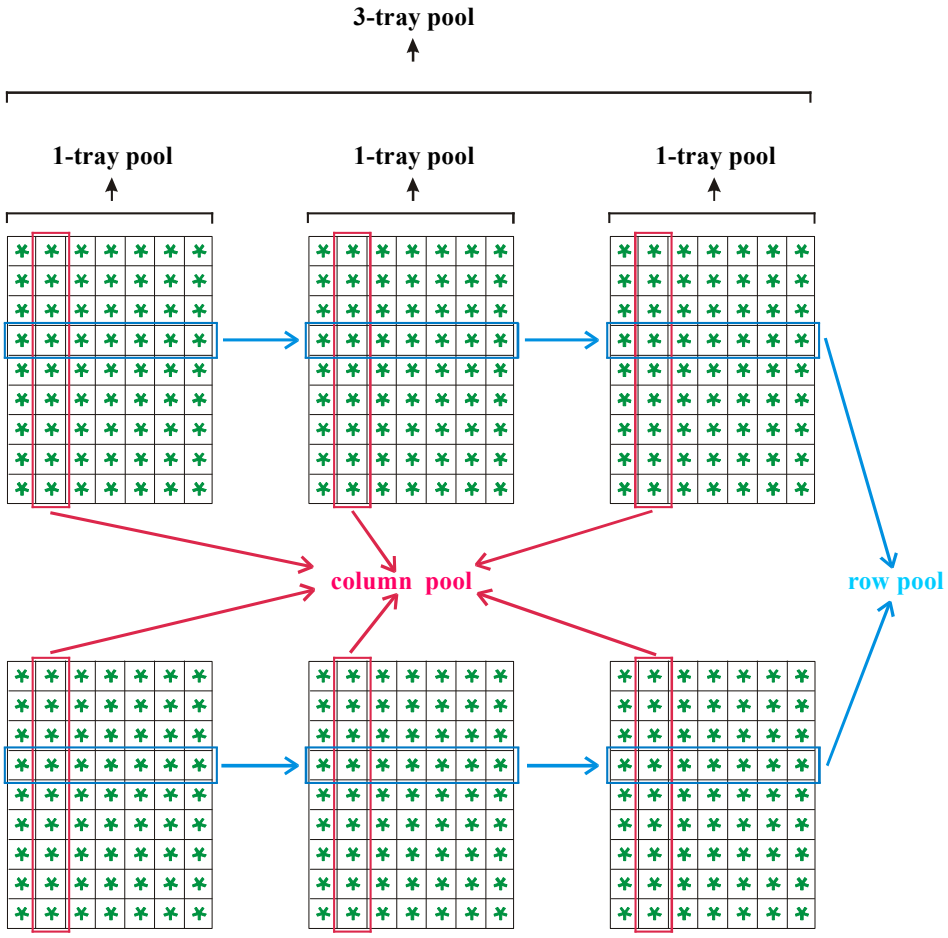

Figure 24: Schematic of 3-dimensional pooling strategy employed in AMAZE

Table 12: Pooling strategy employed in AMAZE collection

3-Dimensional Pooling Strategy of AMAZE	
Pool	**DNA of**
3-Tray Pool	One leaf of each of the plants in 3 trays (sometimes 4 trays)
1-Tray Pool	One leaf of each of the plants grown in one tray
Row Pool	One leaf of each of the plants grown in one row sampled across all trays in one sub-population
Column Pool	One leaf of each of the plants grown in one column sampled across all trays in one sub-population

4.3.2. Principle of the Screen

At the beginning of the screening procedure are PCR reactions using DNA isolated from the 3-tray pools with various primer combinations as *En-1* can be inserted in different orientations. The specificity of the PCR amplification product has to be confirmed by Southern blot hybridizations using a cloned probe of the target gene. Once a 3-tray pool tests positive, the single tray, column and row pools have to be tested to isolate the single plant carrying the insertion of *En-1* within the target gene. If the second PCR reaction results in product, the positives get confirmed by repeating the reactions and adding additional controls with EN primers located at other internal annealing positions within the *En-1* element.

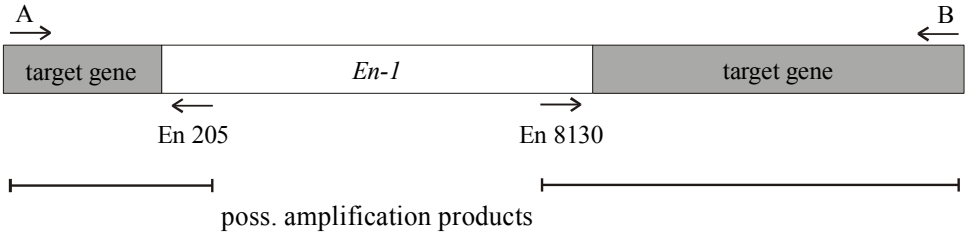

Fig. 25: Primer design used for screening mutant collection

Screening was performed using all five catalytic subunits of protein phosphatase 2A from *A. thaliana* as target genes. The primer pairs for each target genes were designed using the cDNA sequences (see Tab. 13) and the exact sequences are listed in 3.1.6. Gene primers were designed as 26 - 28mers approximately 100 bp into the coding sequence both 5' and 3' of the cDNAs. The expected products lengths were between 0.8 - 2 kb in the cDNAs, however as genomic DNA was used in the screen the actual product could be longer. PCR tests were performed using genomic DNA as template to check the actual length of the products as well as rule out a primer design over an exon-intron junction which would prevent annealing. In some cases more than one primer pair had to be designed to achieve successful target gene amplification (see Tab. 13).

The EN-primers were located at the 5’ and 3’ end of the 8.3 kb long *En-1* element and were directed towards the outside of the gene (Pereira *et al*., 1986; Wisman and Baumann, 1998). As an internal positive control, one primer pair (EN7631, EN8141) had been designed directed towards the inside of the *En-1* element. This facilitated amplification of *En-1* element to be used as control for the PCR reactions during screening.

For the actual screening reactions, all possible pair-wise combinations of gene specific primers with EN-primers were used. Thereby, it is possible to detect *En-1* element insertion independent from its orientation within the genome.

Table 13: Name of target gene primers and EN primers

Primers designed for AMAZE screen [1]		
PP2A catalytic subunit of *A. thaliana*	forward primer	reverse primer
C-1	PC1-1	PC1-2
C-2	PC2-1, PC2-3	PC2-2, PC2-4
C-3	PC3-1, PC3-3	PC3-2, PC3-4
C-4	PC4-1	PC4-2
C-5	PC5-1, PC5-3	PC5-2, PC5-4
En-1 element primers (Wisman and Baumann, 1998)		
	5'	3'
	EN205	EN8130
	EN26	EN7706
	EN91	EN8202

[1] See 3.1.6. for exact primer sequences.

4.3.3. Screening for *En-1* Elements Inserted in Target Genes (PP2A C-subunits)

Before starting the actual screen, different primer tests were performed using genomic DNA from wild-type *Arabidopsis* plants (ecotype Columbia). PCR reactions were performed using single gene primers to rule out amplification products from one gene specific primer alone. Also, the pair-wise combinations of gene specific primers and EN-primers were tested in the absence of *En-1* elements to avoid possible false positives during screening. Additionally, a combination of gene specific primers was used to obtain target gene specific amplification products both as positive control for the quality of the primers as well as for further use as gene specific probes during screening.

4.3.3.1. Testing 3-Tray DNA Pools for Possible Insertions of *En-1* Elements

During the first round of screening, a total of 44 PCR reactions were performed for each pair-wise combination of gene primers with EN205 as well as EN8130 using DNA from 3-tray pools. As positive control, Columbia wild-type DNA was used with both gene specific primers. Additionally, the primer combinations were tested with Columbia wild-type DNA as negative controls. To confirm positive PCR reaction, Southern blot analysis using a radioactively labeled gene specific probe was performed.

Fig. 26 shows an example of a Southern blot analysis screening for the PP2A catalytic subunit C-3. Different pair-wise combinations of the two gene specific primers PC3-2 and PC3-3 and the primers for the *En-1* element EN205 and EN8130 were used as indicated. The strong signal in the third and sixth panel from the top indicated the hybridization of the C-3 gene specific

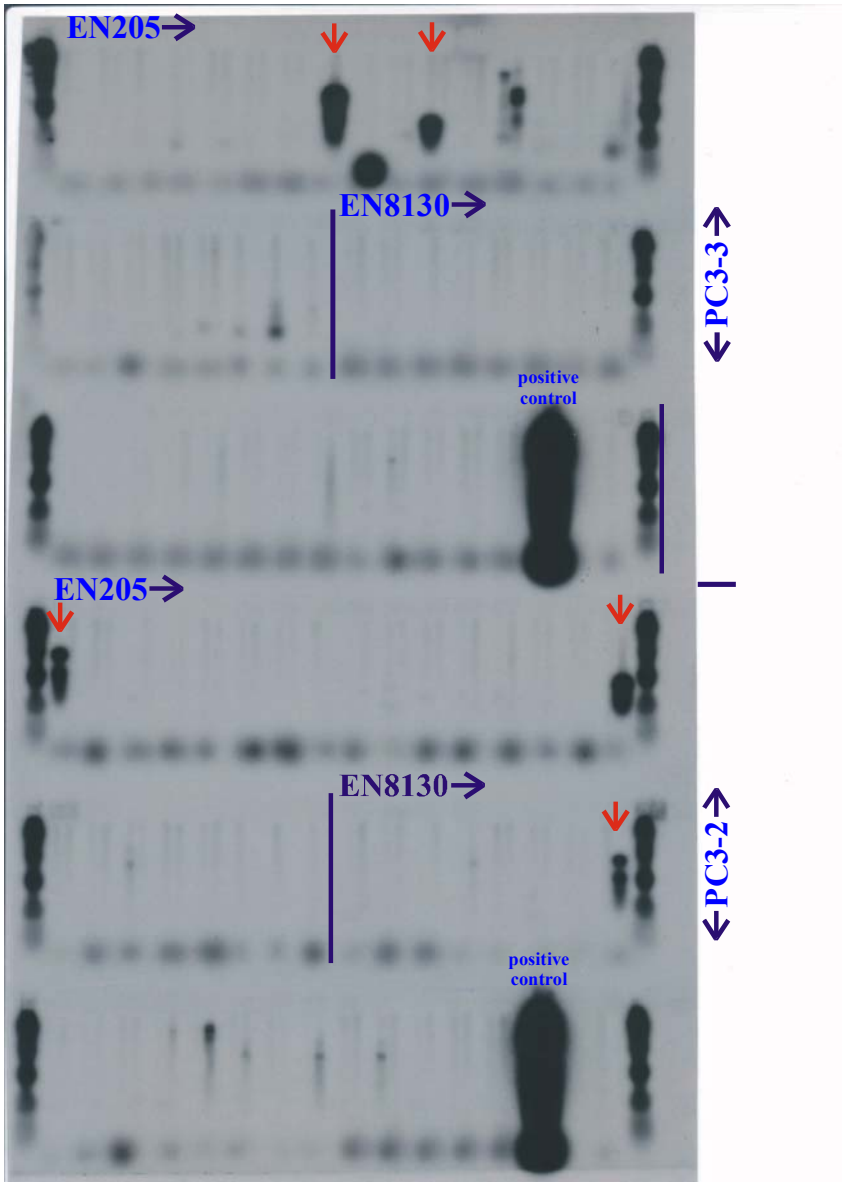

Figure 26: Identification of potential *En-1* insertions in the genes of catalytic subunits of *Arabidopsis* PP2A using DNA from 3-tray pools

Genomic DNA from 3-tray DNA pools was amplified using various pair-wise combinations of target gene primers (PC3-2 and PC3-3) and *En-1* element primers (EN205 and EN8130). A Southern blot was performed using a labeled target gene probe (C-3). The autoradiograph of the Southern blot is shown. Positive hybridization signals are labeled with red arrows.

probe to the amplified product of the positive control (ColWT DNA with PC3-3 and PC3-2). Red arrows denote PCR products showing a strong reaction to the gene specific probe indicating an insertion of the transposable element *En-1*. These hybridization signals are shown by PCR products from 3-tray DNA pools using a combination of one gene specific primer and one EN primer that hybridize to the gene specific probe. The 3-tray DNA pools that showed those signals were further investigated to identify possible positives.

After the first round of screening, several potential insertions were isolated for each PP2A catalytic subunit (Tab.14). The plants from each 3-tray pool were then further tested to confirm and identify the plant carrying the *En-1* element insertion.

Table 14: Summary of potential positives identified in 3-tray DNA pools

A. thaliana PP2A catalytic subunit	Number of potential positives in 3-tray DNA pools
C-1	1
C-2	5
C-3	5
C-4	3
C-5	6

4.3.3.2. Identifying the Plant that Carries *En-1* Insertion in the Target Gene

Upon obtaining a positive PCR signal using DNA from 3-tray pools, it was necessary to confirm this signal and be able to trace it back to one specific plant using DNA from 1-tray, row and column DNA pools. In order to identify the specific plant carrying the *En-1* insertion, DNA from the corresponding 1-tray pools as well as row and column DNA pools were tested using the primer combination that gave the positive signal in the first round of screening. Additionally, DNA from the original 3-tray pool was included to confirm the original positive.

PCR reactions were performed using these DNA templates and Southern blot analysis with the radioactively labeled gene probe was used to identify potential positives. Fig. 27 shows a representative sample of this second round screening performed for C-4. The lower panel showed an unsuccessful confirmation of a positive. The previously observed positive signal could not be repeated using the same 3-tray DNA pool. In the middle panel, the original signal could be repeated using DNA from the 3-tray pool, however, the same signal failed to amplify from any of the 1-tray, row or column DNA pools. The upper panel showed a successful identification of two *En-1* element insertions. The Southern blot showed two different size bands (indicated by red

arrows) within the same 3-tray pool indicating two separate insertion events. The two bands were visible in different 1-tray, row and column pools, thereby identifying two different plants carrying an *En-1* element insertion within the PP2A catalytic subunit C-4. To further confirm the specificity of the *En-1* element insertion, PCR reactions were performed on the identified DNA pools using alternative EN primers. These primers were designed to align at different positions within the *En-1* element sequence. For all positives, an amplification product could be obtained confirming that the original PCR product was obtained by primer annealing within the *En-1* element (data not shown).

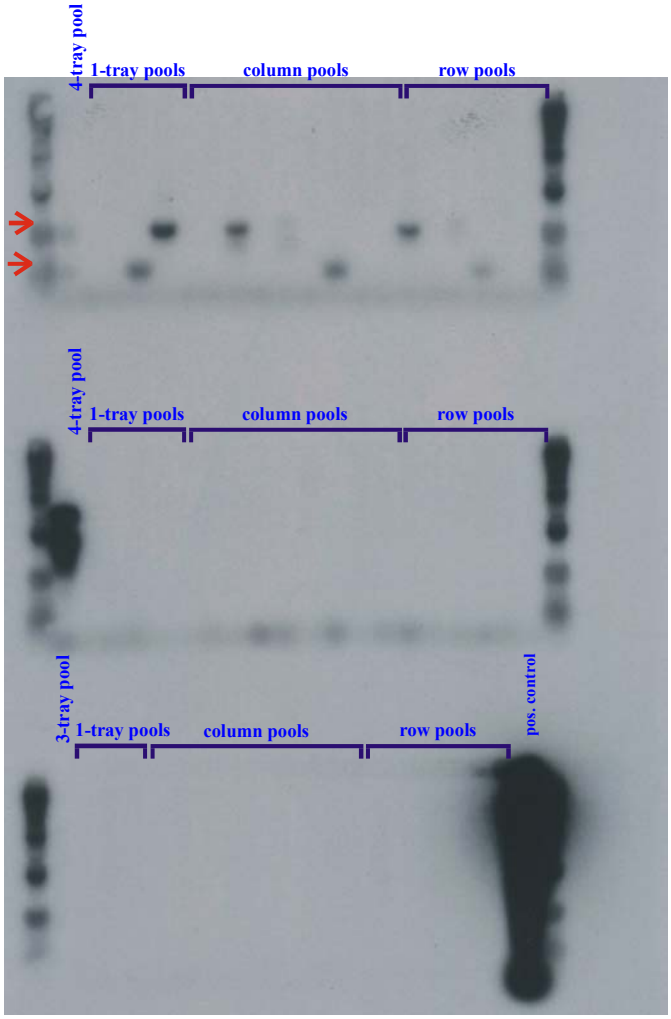

Figure 27: Identification of plant carrying an *En-1* insertion in the PP2A C-4 catalytic subunit gene of *A. thaliana*

Genomic DNA from 3-tray, 1-tray, column and row DNA pools was amplified by PCR using the pair-wise combination of target gene primers and *En-1* element primers that gave a positive signal in the first round of screening (PC4-1/EN8130, PC4-2/EN205, PC4-2/EN8130). A Southern blot was performed using a labeled probe of the target gene (C-4). The autoradiograph of the Southern blot is shown. Red arrows indicate confirmed positives with same size signals in all DNA pools, thereby identifying a single plant carrying the insertion.

As results of the second round of screening, no confirmed *En-1* element insertion could be determined for the C-1, C-2 and C-5 catalytic subunits of protein phosphatase 2A. One insertion could be found with C-3, however none of the 1-tray DNA pools gave a result. Therefore, three different plants were further examined to identify the one plant carrying the mutation and to characterize the insertion. The screening for *En-1* element insertion into the catalytic subunit C-4 resulted in two different plants showing different length amplification products. Both plants were further examined to characterize the expected mutation in the PP2A catalytic subunit C-4.

4.3.4. Characterization of Mutants

4.3.4.1. Identifying F1-Plants that Carry *En-1* Insertion in Target Genes

During the PCR based screening performed at the MPI in Köln, plants that carried an *En-1* element insertion within a catalytic subunit of PP2A were identified. Their seeds were requested to examine possible effects of the mutations within the progeny of these plants. The seeds were kept in the dark for four days at 4º C, placed into soil and grown under normal conditions in the plant growth room. The plants were checked for phenotypes, leaves and seeds were harvested.

Tab. 15 shows a summary of the number of seedlings obtained, number of plants showing sufficient growth to harvest leaves and number of plants that flowered and produced seeds.

An attempt was made to record possible phenotypes. As the screening for insertions into the C-3 subunit did not return the information which specific plant did carry the insertion, all three plants were grown and analyzed. For C-4, two different plants were isolated carrying an insertion of *En-1* in presumably different positions, but within the same gene. Therefore, it was expected to observe a matching phenotypic expression of the gene disruption by the *En-1* insertion. Both plants were grown, but failed to reveal any similarities indicating a common phenotype.

Leaves from plants that also gave seeds were selected and genomic DNA was prepared. Genomic DNA was used as template with the primer pair that gave the original positive result. As negative control ColWT DNA was used with gene specific-EN primer pairs. The specific gene product (C-3 or C-4) was amplified from ColWT DNA as positive control. Southern blot analysis was performed to identify the plants that carry the En-1 insertion in the target gene and compare the result to the previously obtained screening results (data not shown). For C-3 the mutation was identified in the progeny of one of the three possible plants.

Table 15: Summary of F1-generation of plants carrying an *En-1* insertion within a PP2A catalytic subunit gene

mutation in PP2A catalytic subunit		number of plants with			possible phenotypes among plant population
		germinated seeds	harvested leaves	harvested seeds	
C-3 (missing 1-tray DNA pool result - testing plants from all 3 trays)	1	87	63	63	early flowering
	2	78	42	37	very small seedlings (slow growth) some fast growing plants some dark, slightly purple leaves
	3	9	7	7	late and poor germination
C-4 (two different mutations)	C41	50	41	40	early flowering
	C42	132	68	68	some very big plants, some small some dark, slightly purple leaves

However, the fragment hybridizing to the gene specific probe in the Southern blot did not show the same molecular weight as the one identified before. Therefore, it could be assumed that the transposon *En-1* had moved within the same target gene between this plant and the paternal plant that the insertion had been identified. The two different plant lines tested by PCR and Southern blot analysis for the insertions within the catalytic subunit C-4 showed bands of the same molecular weight than observed with the respective parental plants.

For further analysis of possible phenotypes and biochemical as well as transformation experiments, a stable mutant is desired. This could be either a homozygous plant or a plant with a footprint allele which is left behind when the transposon excises and might be enough to cause a disruption of gene expression. To obtain a first indication if the *En-1* insertion is carried in a homozygous or heterozygous manner by the paternal plant, a segregation analysis was performed. For the insertion of the *En-1* element into the PP2A catalytic subunit C-3 only three plants out of 37 still carried the transposon (segregation of 8%). However, it was found again that the original insertion site within these three plants had moved. As only insertions within the C-3 gene will get detected by the PCR based analysis, the experimental design is unable to determine the original zygosity of the paternal plant regarding the insertion within the gene for C-3. For the first identified insertion into C-4 (C41) 20 plants out of 40 tested showed the expected fragment (50%

rate of segregation). The progeny of the second plant identified carrying an insertion in C-4 (C42), showed a 61% segregation rate with 42 plants out of 69 tested showing the expected fragment.
It is expected to see 100% segregation if the paternal line was homozygous, 75% segregation if the paternal line was heterozygous. Experience has also shown that a segregation rate of 67% can indicate that the mutation is lethal in homozygote plants and 25% of all seeds will not germinate or develop into plants. A 50% segregation rate might be an indication for a mutation causing gametic lethality. C41 showed a 50% segregation rate and C42 showed a rate of segregation very close to 67% which could indicate that the homozygous mutation might possibly be pollen lethal or lethal for the developing seedling. However, no conclusive result can be recorded for all three lines tested as the number of seedling tested was low and there is the possibility of the transposon moving which was shown in C-3 and could also happen undetected in C41 and C42.

4.3.4.2. Following *En-1* Insertions into the F2- and F3-Generation

In order to determine the exact location of the *En-1* insertion within the target genes, it was attempted to isolate a plant that carried only the one *En-1* insertion in the target gene within the plant. The AMAZE collection was described to have between 1 - 20 *En-1* elements per plant. To reduce the total number of *En-1* elements in the mutant plant, seeds from plants identified as carrying an insertion within C-3 and from the plants carrying insertions within C-4, were grown on soil and crossed against Columbia WT plants.
Seeds from these crossing experiments were harvested and the seedlings of the F3 generation were again grown in soil. Leaves were harvested from the crossed F3-lines, genomic DNA isolated and PCR reactions using the original primer combinations were performed. No amplification product could be obtained from crossed F3 lines that were made from plants carrying an *En-1* insertion within the C-3 gene indicating that the transposon had completely moved out of this target gene. Within the crossed F3 lines stemming from plants carrying different insertions within the C-4 catalytic subunit, plants that still carried the transposon in the original position could be identified. Using specific gene primers and *En-1* primers the sequence of the PCR product was analyzed to determine the position of the transposon inserted in the gene for the catalytic subunit C-4.
As result of this sequence analysis it was discovered that both C41 and C42 showed an *En-1* insertion within the same gene at different insertion sites. However, the gene was determined to not be the PP2A catalytic subunit C-4 or any other PP2A catalytic subunit. Instead, the plants carried an insertion within a gene on chromosome 4 of *A. thaliana* that was unknown at the time of this study.

Since then the gene has been identified as being a putative SAM dependent carboxyl methyltransferase and S-adenosyl-L-methionine: salicylic acid carboxyl methyltransferase like protein (MIPS code: At4g26420). There a no significant homologies over the whole gene sequence to any of the PP2A catalytic subunits on a nucleotide or protein level, however an analysis of the primer sequence of the gene specific primer PC4-1 revealed a match to the isolated gene at total of 19 nucleotides (see Fig. 28). Out of these 19 matching nucleotides, the 14 nucleotides on the 3' end of the primer showed a perfect match.

Figure 28: Alignment of primer sequence with sequence of target gene and isolated mutant
Red nucleotides show a match between primer sequence and gene sequence. A total of 19 nucleotides could possibly match between the sequence of the isolated putative SAM dependent carboxyl methyltransferase and the sequence of the gene primer with the 14 nucleotides on the 3' end of the primer matching perfectly.

At the time of this study, the *Arabidopsis* genome sequence had not yet been available. Although care was taken to design the original gene specific primers in a way that they would not anneal to the very closely related other catalytic subunits, the chosen primer showed a random match within a totally unrelated gene. Therefore, even though plants carrying an *En-1* insertion within a target gene were isolated, the gene carrying the mutation turned out to be not a PP2A catalytic subunit.

4.3.5. Summary of Screening Results

The PCR-based screen isolated a mutant in a gene for a potential methyltransferase gene. Due to a random similarity between the target gene primer and the isolated gene sequence, a specific amplification occurred in two plants carrying an *En-1* insertion in the gene for a putative SAM dependent carboxyl methyltransferase.

No mutants carrying an *En-1* insertion in *A. thaliana* protein phosphatase 2A catalytic subunit genes were isolated. However, the presence of an *En-1* element within the C-3 catalytic subunit of PP2A could be shown during the screen. Attempting to determine the presence of the *En-1* insertion in the next plant generation, it was discovered that the transposon had moved out of the target gene. Further analysis could be performed to determine if any of the progeny of the insertion carrying plants show a footprint allele that would be sufficient to disrupt the function of PP2A C-3.

Several possible reasons could be responsible for the lack of mutants within PP2A catalytic subunits from this screen. One is the possibility of it being lethal for plants to have mutation in a catalytic subunit of protein phosphatase 2A. Ronne *et al.,* 1991 described knock-out mutants in *S. cerevisiae* catalytic subunits of PP2A. Yeast has two PP2A genes that are very similar to each other (PPH21, PPH22) and a third catalytic subunit (PPH3) that differs in its 5' regulatory site. They could show that a single disruption of PPH21, PPH22 or PPH3 had no effect on the *S. cerevisiae* cells. A double disruption of PPH21 and PPH22 resulted in a severe growth defect, while the disruption of all catalytic subunits was lethal in yeast. There are five catalytic subunits of PP2A that are closely related to each other within *A. thaliana* and the performed screen only attempted to isolate single mutations. Therefore, it seems unlikely that a mutation within one catalytic subunit would be lethal for *Arabidopsis* plants.

Another reason for the inability to isolate mutations within the catalytic subunits of PP2A might be the fact that the mutant plants were grown in soil in all generations. It is possible that PP2A has an important role for seedling development and soil provides more difficult conditions for mutants to grow, therefore implying the possibility of mutation being lethal during seedling development. The results of the two-hybrid screen described in this study showed significantly more possible interaction partners isolated from a library from etiolated seedlings than adult plants, thereby lending credibility to the importance of PP2A in the seedling stage of the *Arabidopsis* life cycle. Possibly, mutants within catalytic subunits of PP2A could have been isolated more easily if plants were grown on plates containing minimal media containing glucose. These plates would enable heterotrophical growth and by removing the need to perform photosynthesis allow plants to grow while carrying mutations within genes necessary for development.

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BCM Search Launcher:
http://www.searchlauncher.bcm.tmc.edu
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Blast-Server:
http://www.ncbi.nlm.nih.gov/BLAST
(National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, MD)

MIPS-Server:
http://mips.gsf.de
(Munich Information Center for Protein Sequences, *A. thaliana* Genome Database, MIPS *Arabidopsis* Group, Institut für Bioinformatik, München)

PubMed-Sever:
http://www.ncbi.nlm.nih.gov/PubMed
(National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, MD)

TAIR-Server:
http://www.*Arabidopsis*.org
(The *Arabidopsis* Information Resource, *Arabidopsis* Biological Resource Center (ABRC), Ohio State University, Columbo, OH)

6. Appendix

A. Two-Hybrid Screen: DNA Sequences of Isolated Proteins

Sequence data converted using readseq at BCM Search Launcher/Sequence Utilities

>H17a, **C1-1**, 769 bases, D97 checksum.
TAAAGAAGGACTCGAGGCTTCTCAGAGAGTCCAGATTGCTCAAACCCGAG
CCAAACTGCTAAAAGAAACCGCAATGGAGAAGCAGAGAACCGTAGACTCG
GTTTTCGCAGCAGCAAAGACCACTGCTCAAAGAGGAGACGCGTTGCACAT
CAGAATCGTAGCGATCAAGAAACTGTTGGCAAAGCTAGAAGCAGAGAAAG
TGGACGTTGATTCAAAGTTCACCTCTTTAACGACGAGTCTGTCAGAGCTT
CTCAAGGAGGCGTCACAGGCTTACGAAGAGTATCACGAGGCGGTGCATAA
GGCAAAGGACGAGCAAGCGGCTGAGGAATTTGCGGTGGAGACGACAAAGA
GAGAGCAGAACATATTTGGGTTGAGTTTCTTAGTTCACTTAATTGAACTA
ATGAGATTCTTGATTGTGGTTAAAGCACATGTTCAATTAGTTGTGGTTCT
TGTGTTTTATTTTCTTGTGTTGGTTTGTTTGAGACTTTGTTTGTTGCTTA
TATCAGGGGAGAGAGGTTGAAAAACCTTTGAGAGAGATAGATTAATGAGA
GATTTGTGAAACACAAATAATGTTTTAGTCGTCTTATAATAAAACGGTAA
ATCTCGAGAGATCTATGAATCGTAGATACTGAAAAACCCCGCAAGTTCAC
TTCAACTGTGCATCGTGCACCATCTTCAATTTCTTTCATTTATACATCGT
TTTGGCTTCTTTTATGTAACCTATCCTCCTCNTAAGTTTCAATCTTGGGN
CATGTAACCCTCTGATCTA

>H18a, **C1-2**, 774 bases, 143A checksum.
TAAAGAAGGACTCGAGGCTTCTCAGAGAGTCCAGATTGCTCAAACCCGAG
CCAAACTGCTAAAAGAAACCGCAATGGAGAAGCAGAGAACCGTAGACTCG
GTTTTCGCAGCAGCAAAGACCACTGCTCAAAGAGGAGACGCGTTGCACAT
CAGAATCGTAGCGATCAAGAAACTGTTGGCAAAGCTAGAAGCAGAGAAAG
TGGACGTTGATTCAAAGTTCACCTCTTTAACGACGAGTCTGTCAGAGCTT
CTCAAGGAGGCGTCACAGGCTTACGAAGAGTATCACGAGGCGGTGCATAA
GGCAAAGGACGAGCAAGCGGCTGAGGAATTTGCGGTGGAGACGACAAAGA
GAGCAGAACATATTTGGGTTGAGTTTCTTAGTTCACTTAATTGAACTAAT
GAGATTCTTGATTGTGGTTAAAGCACATGTTCAATTAGTTGTGGTTCTTG
TGTTTTATTTTCTTGTGTTGTTTTGTTTGAGACTTTGTTTGTTGCTTATA
TCAGGGGAGAGAGGTTGAAAAACCTTTGAGAGAGATAGATAAATGAGAGA
TTTGTGAAACACAAATAATGTTTTAGTCGTCTTATAATAAAACGGTAAAT
CTCGAGAGATCTATGAATCGTAGATACTGAAAAACCCCGCAAGTTCACTT
CAACTGTGCATCGGTGCACCATCTCAATTTCTTTTCATTTATACATCGGT
TTGGCCTCTTTTATGTAACTATACTCCTCTAAGTTTTCAATCTTGGGCAT
GTAACCTCTGATCTNTANAAATTT

>H19a, **C1-3**, 762 bases, 1C46 checksum.
GCGAGAAGGAATTCGAAGAAACAGATTTCAACAATGGCGGCAGTTCAACA
GCAACAAGCGATGCAGAAGAACACTTTATACGTAGGAGGTTTAGCTGATG
AAGTTAACGAATCGATCCTTCACGCCGCGTTTATACCTTTCGGCGATATC
AAAGACGTGAAGACGCCGTTGGACCAAGCGAATCAGAAGCATAGATCCTT
CGGATTCGTTACTTTTCTTGAGAGAGAAGACGCTTCTGCTGCTATGGATA
ATATGGACGGTGCTGAGCTTTATGGTCGTGTTCTCACTGTTAATTATGCT
CTCCCTGAGAAAATTAAGGGTGGTGAACAGGGTTGGGCTGCTCATCCACT
TTGGGCAGATGCAGACACATGGTTTGAGAGGCAGCAACAGGAGAAGGAGA
TTCTGAAGATGCAAGCTGAGAACAAGGCTGCCATGGAGACTGCTGAGGAG
CTTCACAGGAAGAAATTGGCGGAAGATCGACAAGGAGAGATGGAGGAAGA

TACAGATACCAAAAATGATCCTATGGCCAGAGCAGAGGCTGATGCTCTAA
GTCACGGCGATGCCTAATGAAAGTAAGCGTTACCGGATTCTCTTATGCAC
CTTGTCCTGTAGGATAACAAGTATGTTTTCGCCTGAGTGTGNAACACTGT
CACTATCACCTCATAGAGTTTTGAGGGTAGAATNGTTCTTGANGNTGATT
ACCATGTTATTGCTATTAAAAGGTAAATCTCGAGAGATCTATTGAATCGT
AGATACTTGAAA

>H20a, **C1-4**, 766 bases, 1CE7 checksum.
GTTCAGTGATGTTTATATCTCTACTGAATTAATGGGATACTGGATCTTCA
TCAAATCATCAGATCTAACCCAGAAGTTTATCANAAAGAAACACTGTCAG
TACTTCTTTGTACCAGCCTACTTCCGAGGACTTGGAAGTATATCCCACCT
CAGGCTTAACATTTAATCCATAGGGAATTTAAANCCCGNNCAATTCCTTC
TGTTGAACGCCGAATTGCGATTTTAAAGATTCGTGATTTCGGTCTTGCTA
GACCCNCTTCANAGAATGATTTTATGACCTGAGTATGTTGNTACCGAGAT
GGTATAGAGCACCTGAGCTTCTGTTGAACTCTTCAGAATTACACAGCTGC
TATTTGATGGTTTGGTCTGTTGGTTGTATCTTTATGGAGCTTATGAATAA
GAAAGCCTTTGTTCCCTGGTAAAGACCATGTTCATCAAATGCGCTTATTT
GACAGAGTTGCTTGGCACACCGACAGAATCTGATCTCGGGTTTTACTCAC
AATGAGGATGCGAAAAGATACATCCGGCAACTTCCCAACTTCCCACGTCA
GNNCCTTAGCTTAAACTTTTCTCTCATGTTACCCCAATGGNCATTGATCT
TTGGTGACAAGAATGTTTGACCGTTTGACCCCAACCAGAAAGAATNACTT
GTTGGAACCAAGCTCTTGAATCACCAGTACCTTGCTAAATTTGCACGAAC
CCCGAATGGGNGAANCCCANNTNTCCAAAAACCCNTTNCTTTTTTGNGGT
TNCAACCAACAANCCC

>H21a, **C1-5**, 775 bases, 26F2 checksum.
CTTTGTCCATAATGGGAAGCTACACAAATATTTGACTGTTACAGATGGCT
TAGTGAAGCGATATAACAATGCGAAAAAAGAATGGGGTTTCGGACAATTG
ATTCCTCGATCAACATTCTACAACGCGAACGAAGGTTACCTTGACCAGGA
CACTGGTTCTTTTGGTGCTGAGATCTTTATTGTTAAACCGGCTCAACAAC
AAGAGAAAGTTACATTCATATCAAACCCTCCAAACAATGTTTTCACTTGG
AAGATACTTCGTTTCTCTACCTTGGAAGATAAATTCTATTACTCCGATGA
TTTTCTCGTTGAAGACCGATACTGGAGACTAGGATTTAACCCGAAAGGGG
ATGGAGGAGGAAGACCACATGCACTTCCAATCTTNCTATTTGCTCAAGGC
CATAAGGCAAATGCAGTTGCTACAAACACTTGGGGAGCGGTTAATCTGCG
GTTAAAGAATCAACGAAGTACTAACCATAGACAAATATATTCTGCAGCTT
GGTACCCGATTGGAAGCGGTTATGGTGTGGGAGTGAACAATATCATACTG
TTAGCTGATTTAAACGATGCATCANAAAGGATATTTGGTGAATGATGCCA
TTATCTTTGAAGCTGAAATGGTTAAGGTCTCTATAACCAACATCGGCTCC
CGCTTAAANATCTGCACCTTCTTTGNCTAACGATCAATCAAACCTTATGA
ATAAAAAAGATGGTNTAAATTTGAATGAGNTTTGGGATANAGAAAACNGT
TNATGTTTTGTGAAATGTGGANTNN

>H22a, **C1-6**, 782 bases, 561 checksum.
CACGCCAGAAAAGCCCAAAGAACCCGAAAAGACGAAGCAACCCGCACCCG
CTCCAGCTCCCGCACAAGCACCCGCAGCCAAACCCGCACCCGCTCCCGCT
CCAGCTCCCGCACCTGCCCCGAAGCAGCCGGGACCGCCACCGCAAGCGAT
CCCGATGATGCCGCAAGGGCAGCCAGCAATGTGTTGCGGGCCCTACTACG
ATGGATACGGAGGGCCAGCATTCAATGGATATGGAATGCCGCCGCAGCCT
TACGAGTGCTATGGACGACCAGTCTACGAGAGCTGGGGTGGGGGCTGCCC
ACCACCACCACCTGCTTATAGACAATGCCACGTCACTAGATGTGATTACT
TCAGCGAAGAGAATCCACAGAGCTGTTCCATCATGTGAATTTGACTTCTT
TATCTCTAAATCCTTTATTTTTCCTTTAATCTTTTTTTCTTCTTATACAA
ATAATAATTAAAAAAATATAGAGATGTTTTACGTGTAATGTTGTGATTAA
GAGAACCAATAAGGAAGGAACGTTGTATTTTGTAAATGGTAGCCATTTCC

AATATTTGGTTGTCCATTCGAAGCTGTAACTGTAAACACTATAAAGAAAA
TTTGGTAAATCTCGAGAGATCTATGAATCGTAGATACTGAAAAACCCCGC
AAGTTCACTTCAACTGGGCATCGTGCACCATCTCAATTTCTTTTCATTTA
TACATCGGTTTGGCCTTCTTTTATGTAACTATACTNCTCTTAAGTTTCAA
TCTTGGGCATGTAACCCCTGANCTATAGAAAN

>H23a, **C1-7,** 774 bases, 1BED checksum.
CTTTGAAACTCAACATGGTTGCTGTTGATCAGGTGTATCCGTTGTTGTCT
GATCTCTCGGCTTCTCTTAACAAACTAAGTATATTGCCACCAGATTTCGA
GGGGAAGATTAAGATGAAGGAATGGCTTTTGAGGCTGTCAAAGATGGGAG
CTTCAGATGAACTCACTGAGCAGCAGGCTCGGCAACTTCACTTTGATCTT
GAGTCATCCTATAACTCGTTCATGGCTGCTTTGCCTAATGCTGGTAATTA
ACCGATGTGCCACTAGAGACTGATGTGTGTTCGGTTTATTTTGTTTAGTT
TGGCTTTATGTAGTTATAATGATGGCTTTAGGAACAAAGATTTTTGTTTC
ATGACAATTTGCTACATATATTTTGGGGATGAATGTTCGTAGACAAAACA
AGCTTTTCCTTTTGTTGTGTAAAAGATGATGCCAATCCAGAAATTGTTTT
TTTCATGAAGTGTAATGTATGAATCTAACTCTCTTGTGCTGNAGAAAANA
NAAAAAANCGNGAGANNTTANAGCANGNANNNCCTNTTAGAGNNNNGANG
ATTANNNNCGNANNAANATAANANTCGGGGNNAATNCNGNNGATATTTNT
NTNNNGNAANCNNTCATNAAAACCCCCCGGGGNTTTTTATTNGGGTNGGG
GGGCNCCNCNAAANATTTTTTTTTTAAAACACGGGGGGNCCNNCTTTTGN
NAGAANCNCCCCCCCCCAAAANAAANAAAATNGGGGGCNGGTNNCCCNTGT
TANTAAAAAAATTTTTTNAAAANN

>H24a, **C1-8,** 764 bases, 1C81 checksum.
TTTTTTTTTTTTTTTTTTTTTGGGTTTTTTTATTATTCATTAAAATTTCC
ATGTCTCGTAAATTATGATCTAAATTAGTTAGGGCTCTTTAAGTAAAATA
AACAATAACAAAAAAATTCATTTTCATAATTCACATTCCCTGGTTTTCTC
AATTATAGTTATTAAACCGTACAATACAATTGCACAAAAGCCTTCCAGAG
ATATTACAACTCCAACAACAAACCCAAAGACACATAAATGATCAATCAAT
AGACAAAGATTCATCTAAAAATGATCCAACGGCCAGAAACCCATTACGAT
GCAAATTAAAAAAGCAAATCACGAAAAATTCATCAGATTAACAACGTTGA
ATTTGACGTCTTCAGTAATCGGTGGTAGGCAATTGCTCGTGGGCATTTTC
ATCGATGAAGACAAAGTCGTAGATAATTCCGGCGAGTCCACCACCAATAA
GAGGACCAGCCCAGTAAACCCAGTGGTTGGTCCACGTCCAGCTTACGACG
GCTGGTCCGAAAGCAACGGCTGGGTTCATGGAAGCTCCGCTGAAAGCTCC
ACCGGCGGAGGATGTTAGCTTCAACGATGAAACCTATGGCGATTGGTGCG
ATTGTTCCGAGACTACCGTTCTTGGGGTCAACAGGCTGTGGCGTAAACGG
TGTATACGAGCCCGAAGGTCATCACGATCTCGAAGACGANAGCGTTTAAT
GATCCGCTTNCGGCAGANAGACCCGAACGCTGGAATTGGCTCGCCACCGN
GGGGAAAGNTTANG

>2a, **C3-2,** 174 bases, 2027 checksum.
GAGAACTAAAATGATGACCTCATCTGTTAAAGTAGAAGAAGATAAAGAGC
GTAATGAAGATATTACAGGAGATAACAAATCTTGATTACTTGTACCAAAG
AGCAATGTTTCGTTTTTTCTTTCAATAAACAAAATGTCTCAAAGCACTCG
TCGTTCTAAGCTTCATTCCCTTGG

>H2a, **C3-3,** 597 bases, 19F9 checksum.
CGCGATTCAGCCTTTCGGTAAAATCCCAGTCCTCGTCGACGGAGACTACA
AAATCTTCGAGTCGCGTGCGATCATGAGGTATATAGCAGAGAAGTATAGA
TCACAAGGACCTGATCTTTTGGGGAAGACTATTGAAGAGAGAGGACAAGT
AGAGCAATGGTTAGACGTTGAGGCTACAAGTTACCATCCACCACTATTGG
CTTTAACGCTCAACATTGTCTTTGCACCACTTATGGGTTTCCCTGCTGAT

GAGAAAGTTATTAAGGAGAGTGAAGAGAAGCTTGCAGAAGTGCTTGATGT
CTATGAAGCTCAGCTTTCTAAGAACGAATACTTGGCTGGTGATTTTGTGA
GTCTAGCTGATTTGGCTCACCTTCCTTTCACCGAGTATCTTGTTGGTCCT
ATTGGGAAGGCTCATTTGATCAAAGATAGGAAGCATGTTAGCGCTTGGTG
GGATAAGATTAGTAGCCGTGCTGCGTGGAAGGAGGTTTCCGCTAAGTACT
CACTACCTGTTTAAACAAGGAGATGTTGACTTGGGGGGTTTGTGTGAGCT
TTGTGTTCTTTGAGGGAGTTCGTTTCGGGTCGATTGTCCTTTGGGGG

>4a, **C3-4**, 151 bases, DE5 checksum.
GGCGTTCTTCAACAAACGTTCTATTCTCTCTTCAATTCCAAGCTCCCACC
ATCCTCCTTCCAAATGGCCAGATCTCTCCCACTCCGAAACCTTCCCAATC
CGAATCAACAACGGTGGAAAGGCCGGAGCAAGAATGTAGCAGTGATAGTG
G

>5,**C3-5**, 247 bases, F1C checksum.
CAGATCTAAAGTTGAGTCTGAGCTTTCTTCTGTTTGCTCTGGAATCCTTA
AGATCCTTGACTCGCATCTGATCCCATCTGCTGGAGCGAGTGAGTCTAAG
GTCTTTTACTTGAAGATGAAAGGTGATTATCATCGGTACATGGCTGAGGT
TAAGTCTGGTGATGAGAGGAAAACTGCTGCTGAAGATACCATTGCCTCGC
TTACAAAGCAGCTCAGGTATACGCACGTGCGATAGCACCTACACGTA

>6, **C3-6**, 252 bases, 8F0 checksum.
GGGAAATATTGGAAAGAATACAATGGTGGTTCAAGGTAGCACCTACTGTG
ACATTTGCAAATCCGGCTTCGAGCCTCCTGAATCCTCCTACTTCATCCCC
GGTGCAACGGTGAAGCTATCATGCAAAGACAGGAAGACAATGGAAGAGGT
TTACACAGACAAAGCTGTATCAGACAAAGAAGGAAAGTATAAGTTCAATG
TCCACGACGTCACAGAGACCAGATGTGCGATGTTTGCTTGTGAAAGCTCG
AT

>7, **C3-7**, 218 bases, 1386 checksum.
TTTTCTCCTCCATCAAAATCCTCCGACGATGACAACCATAGCTGCAGCTG
GCCTCAACGTCGCGACTCCACGATTGGTCGTTCGACCTGTGGCTCGTGTA
TTACGTCCGGTCCGGTTGAATTACCCGTGGAAATTCGGTACGATGAAGCG
GATGGTTGTGGTTAAGGCTACACGGAAGGACAGATATCGGAGAGGTGGAC
AAGAGTATACAAGAGACA

>H3a, **C3-8**, 607 bases, 97B checksum.
CAGAGAGATTCATGGCGGAAGCTTGCGGAGTGAGAAGGATGAAGCTGGGA
AGCCAAGGCCTTGAGGTATCTGCGCAAGGCCTTGGTTGCATGGGCCTCTC
CGCTTTCTACGGTGCTCCCAAGCCGGAAAATGAAGCCATCGCTCTCATCC
ACCACGCTATTCACTCCGGCGTTACTCTCCTTGACACCTCCGATATCTAC
GGCCCTGAGACCAATGAAGTTCTCCTCGGAAAGGCTCTCAAGGATGGGGT
GAGAGAAAAAGTGGAACTTGCGACCAAATTTGGAATCAGTTATGCAGAGG
GGAAAAGAGAGGTCAGAGGAGATCCTGAATATGTAAGGGCTGCTTGTGAG
GCAAGTTTAAAGAGGCTAGATATTGCCTGCATCGATCTCTATTATCAGCA
TCGTGTCGATACTCGTGTCCCTATCGAAATCACTATGGGAGAACTGAAGA
AGCTGGTTGAAGAGGGTAAAATAAAGTACATTGGTTTGTCTGAAGCCTCT
GCCTCAACTATCAGAAGAGCACATGCTGTTCACCCAATTACTGCCGTGCA
GATAGAATGGTCCTTGTGGACGAGAGATGTGGAAGAAGAAATCATTCCCG
ACCCCCC

>9a, **C3-9**, 113 bases, 769 checksum.
CTTCCTCCTCCACGTCGCAACCACCTCCTCCGCCGCGATTTCCTCTCCCT
GGCGGCAACATCCACGCTTCTAACACAATCGATTCAGATTCTAGCGTCGG
CGCCGGTCTCCGA

>H4a, **C3-10**, 599 bases, 1D25 checksum.
TTTTTTTTTTTTGAAATCATAATCATCATACATAATATTATCTGATTGCG
TATTCACATTTGGTGTCCACATGGGATGAATATTGTCCACCACCACTATT
ATTTATATTCACATATGCATTATCAACATTTTAACGCCACCTGTACAATC
CCAACCACTAGACGTTAAAATTATCCAACGGCCACGATCCGCCCACTCGA
TCACGTCACGCACCGACGGCTGCTATTGAAGCCATACCCTCCAGCTCACT
CTTGCTCCGTCTGATCCTCTCACCACCAACGTCAAATCCGCCTGTCGCCG
TCGCCGTAGCCCAAATCTCTGGCACCGCCGTAGCTATCTCAGTAACCGTC
TCCACCGCATAATCCGCATCTTTCGTCTTTTCTGCTCTTCCCACCAGGAT
TGTGCGAAGCCCGACAGATTTACCCGCGGTAATATTGTGGATATTATCAT
CCAAAAATACTGTTCGGCGAGGATCAACGTTGGCTACACGAATGCAAATA
TCCATTGCGGTCAACGAAGGCTTGAGAACAACCGGGTATTCATCGGGTCG
GGTTGTTGACCCGAATAAATTTGGGTTCATTGTCTCAAAAACAAAAAAA

>11, **C3-11**, 205 bases, 1C34 checksum.
GAGAGCATTTTATATGTTGAATCATCTTACGCAGGCTGTTGTAGCAGCAG
TGTCTATCGGAGACGATGAAGTTCAACGTCGCCAATCCGACCACCGGGTG
TCAAAAGAAACTCGAGATCGATTGACGATCAAAACTCCTCATGCTTTTCG
ACAAGAGGCTCTCTCAGGAAGTTAGCGGAGATGCTCTAGGCGAGGAATTC
AAGGT

>12, **C3-12**, 191 bases, 95F checksum.
AGAATAAAAAATTTATATATAACTTTTCAGAAAGCAAAAAAAAAACAAAG
AAAGGAAAACTCGAAACAATGGTTGATGGTACAATACACATACTATTGCA
TTTGGTTATTTATTATATAGATATATATATCCATCGGAGACTGTTTATGC
ACTGTCGACCGCCGCTCTGCTGCTTCATGCTTCTTCCTGCG

>13, **C3-13**, 187 bases, 99E checksum.
AGTTCACCTCAAACTCTAAAATCAATCTCTCAAATCTCTCAACCGTGATC
AAGATGCAGATCTTCGTTAAGACTCTCACCGGAAAGACTATCACCCTCGA
GGTGGAAAGCTCTGACACCATCGACAACGTTAAGGCCAAGATCCAGGATA
AGGAAGGTATTCCTCCGGATCAGCAGAGGTTATCTTG

>14, **C3-14**, 183 bases, F4 checksum.
TCTGTCTCTAACTCTCGGGAAACAAATCATCGACGAGTCTTGCTCTCTAG
GAAAATCTTTAACCGATAAAAAGATTCAAACCCTAGCTTCGAAAGGAGAC
CAACGCAACGCCATCAACGTCGTTTTGACTTCAGCTGACGTTACGGTTAC
AGGATTTGGTATGAGTCGTTGCGGACTCACGGA

>15, **C3-15**, 185 bases, 7AA checksum.
CACAAAACAAAAAAAAATGGCTTCCACTGCTCTCTCAAGCGCCATCGTCG
GAACTTCATTCATCCGTCGTTCCCCAGCTCCAATCAGTCTCCGTTCCCTT
CCATCAGCCAACACACAATCCTCTTCGGTCTCAAAYCAGGACGTCGTGGT
GGACGTGTAAGCATGGTACATACAAGGTAGTTATA

>16, **C3-16**, 175 bases, 415 checksum.
AAAGGACAGAGTCAAGCCATCTCCCACACCTGATAGCTGGAAGCTCAAAG
AAATTTTCGCCACTGGAATTGTGCTGGGAGGCTACCAAGCCATTATGAGT
GTTATTTTCTTCTGGGCTGCTCACAAGACCGACTTTTTCTCGGACAAGTT
CGGTGTGAGGTCAATCAGGGACAAT

>H5a, **C3-17**, 605 bases, 1F48 checksum.
TTTTCTCTGATTCTTCTACTCTTTCCTAATTTACCGATTTTCTCGGGAAT
CCAAAATCCCGTTTCTCTTTTTCTCGAGTTAAAAAACTTTCGATCTGAAG
TCAATTCAGATTCTTCTTGGAAGCTCAAAGAAGAAGAAGAGAGAAGAGAG

AGAGACTAGTGATTTGGTTTAGTTCGTTGGATCAGAAAGGTCTCTGCTTT
GATCTTTGTAGTACACGTGGCGAAAAGGGTCGGAATTTGGAGTTGTGTTG
ATGATGTTCCAAGTCTCAGATTTCTTGTAATAACATTCTCTTGTCTTTCT
CCAATGTCTTTGTCTTGATTCTCAGAAAAAGATCTTCAGATCCGTTTACT
TGAGGGGAAAGAGACGAATAGTTTTTTGTTAGCTAAAAAAAATTATGATG
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TGGCGGTAGAGGTTCCGGCGGCGGTGGTGGTGGCGGCGGCAGGGAGTCAG
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>H6a, **C3-18**, 599 bases, 341 checksum.
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ACTTTGACAAAGCTCGTAAGACTCGAGCACCCGGTAGCAAGATCATGAAC
CCGAATGACCCGGAGTATAACTCTGACTCTCAATCACAAGCTCCTCCTCA
TCCTCCTTCTTCCAGAACCAAACCTGAGCAAGTTGACACGGTTAGAAGAT
CACGTGAGCATATGAGAAGCCGAGAAGAGAGCGAGTTGAAACAGTTTGGT
GATGCTGGTGGTTCATCAAATGAAGCTGCTAACAAAAGACAAGGAAGAGC
TTCTCAGAACAATAGTTATGACAACAAGTCACCTTTGCATAAGAATTCTT
ATGATGGTACTGGAAAATCTAGGCCTAAACCTACCAACCTTAGAGCTGAT
GAAAGTCCTGAAAAAGTCACAGTGGTGCCTAAATTCGGTGACTGGGACGA
GAACAACCCGTCATCAGCTGACGGATACACGCATATCTTCAATAAAGTCC
GTGAAGAGAGAAGTTCTGGAGCAAATGTGAGTGGATCTTTCAAAGGGAA

>H7a, **C3-19**, 611 bases, 7BE checksum.
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GGTAAAACGATGAGGGAAATCTAGAAGTCTCTAGAGAGAGATGACAACCA
GATTATTCGATAATGAAAGCATGAGCATAACATGTTGCTCTTATACAAAA
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CCATTTGCATTGCCAGCTCGCCAGCTCAAAACACGTGCTTTGCTTAACAT
CCCTGAGTTCACATTGCCATCTCGCCAGCTCACAACACACACGTGCTTTG
CTTAACATCCCTGGCCAAACAACTTAGCAACAATTGCTCCATCCTCTTCC
GTTGCTCCTGTGCCAAGGCTCATAACTATATACACACAAACATAATTGGC
AAATGTAAGCATTTGACAGCTTCAGAGAGAAACAGAAATCACAAACAACA
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GACTTCACATTTCCAGCTCGCCAGCTCACAACACGTGCTTTGCTTAACAT
CCCCNTTTGGG

>H8a, **C3-20**, 600 bases, 1D82 checksum.
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AAGACGCTGATCAGGGGAAAGATCCAATGCGTAGATCTTTGTCTACTGCA
GATGGGAACAGAAGAGGAGAGGTCGCAATGGGGAGAATGAGTAGGGACTC
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ATGCCTGGGGAAATGAGAGACCAAGGAGTTACAGAAACAAGACTCCAACT
GCTTAAAGGTGTGACTGGTGCATTTAGGCCAGGAGTTTTGACTGCGCTTA
TGGGAGTGAGTGGTGCCGGTAAGACTACGCTTATGGACGTTTTGGCCGGA
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CAAAGGTTCAAGAAACATTTGCTAGAATCTCAGGATATTGTTGGNAAGGG

>H9a, **C3-21**, 606 bases, B55 checksum.
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AGAACAGATTGAATCTGATGAAGCTAAGGAGAGAAAGTGGAAAAAATCTT
CAAGATCTCTCCGGAAAAATTCTTCTGAAACTAAAGAATCCGCCGCCGGA
AACACCGTACGGACAGGAACTCTCGTCGTGGCTTAGTAGAAGCTTCAGTT
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AAGATTTGCTCTGTTTCTATTGATCTTTCTATGGATCGGAGATAACTAGT
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TTTTTT

>H10a, **C3-22**, 755 bases, 22B3 checksum.
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GTTCTTCCTTCAAGCTATTCTCCATCCTCATATCTTGTTGAAGGCAACAA
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TCGATCATGGGGTCGATAGATACAAGGTCATCAACAATGGTGCACATGAT
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TAACAGCTTTCTTTTCCTCTTCGGTCTCCTCCCCGAAAAGGNCAACATCA
TCATCATCTTCTTCTTCAGCANCAGTATCCTTANAATCAGCTGCANGGGG
AGTNGNAACAGGCCTCTTCTGGGATANGNGATGACCCTTCAACAATTGAC
CCANCTTTCTTTNAGNAAGAAGACAACCCANANATCCTCAANAAAGGGCA
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AGTTG

>H11a, **C3-23**, 758 bases, 1239 checksum.
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GACCGCTTTTTGGTCTGATGAGTTTCAGCTTTGTGGCGACGGTGACTCTG
TTTCTGATCGCTTCTCCATTGCTGCTGATATTTGCTCCAGCTTTTATGGT
AACGGTAGCGGTTCTTGTGTCGGCTATGGTTGGTGTTGGGGTAGCTGCAG
CCATGTGGATGATGGGAATAGCCGCTTTGGTATGTTGTGGCCGAGAGATT
GGTATTGAAACCGGACTGGCTGGGAGGATGGTTGAGTCGGTGGTGAGAGA
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ATTCAACTTCTTCTCCTTCTTCTCGGGATTACTCTTCTTCTTAGTTCAAA
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AGAAACAAAAAAAAAACAGAGACATGGCTATGATAAGAAAACAAAATTAAG
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AGTAATGCTATATCAATTTGGTAAAATCTCCGAGAAGATCTATGAATCGT
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TAACTATT

>H12a, **C3-24**, 742 bases, 6F7 checksum.
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GCACCATCTCAATTTCTTTCATTTATACATCGTTTTGCCTTCTTTTATGT

AACTATACTCCTCTAAGTTTCAATCTTGGCCATGTAACCTCTGATCTATA
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TTCTTCATGAAAATATATTACGAGGGCTTATTCAAAAGCTTTGGACTTCT
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CCTNTTACCGGCATGCCGGTCGAGGGACCTAATACTTCGTNTAGGATACA
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>H13a, **C3-25,** 753 bases, 1393 checksum.
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ACAAGAGAGTTGAGAGACGAGCGACCATCAAGTAAGATAGTGACAATCAC
AAGCTTCTCTGTGATTAAGGACAGAGGAGAACCCTATGAATCATCTATTT
TTGAGGCTGCCGGTTACAAATGGAGATTAGTTTTGTACGTGAAGGGTAAT
CCGAAAGGCGGTATAAATAATCATATTTCACTTTACGCGAGGATAGAAGA
GACAGAAACTCTTCCAAGAGGGTGGGAAGTGAATGTTGATCTCAAACTCT
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TTCTCTTCCAACATTCTACAACGCGAACGAAGGGTACCTTGTGCAGGACA
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>H14a, **C3-26,** 735 bases, E0 checksum.
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TTTCATTAGGAACAATATGAGCGACGTTGTGAGACCCGAAGGATGGCTTG
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>H15a, **C3-27,** 753 bases, 13A2 checksum.
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TGACTTTGACCCACTTGATGTGACCAAGATCTGGCCTGAGGATATTTTGC
CTCTGCAACCGGTTGGTCGCTTGGTTCTGAACAGGACCATTGACAATTTC
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>H16a, **C3-28,** 790 bases, 94A checksum.
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B. AMAZE Screen: Sequence of Gene carrying *En-1* Insertion

At4g26420

S-adenosyl-L-methionine:salicylic acid carboxyl methyltransferase-like protein

```
   1 aaaaaccaat ggacagcaaa atatggaaaa tctcagctca gcagtttcac taattcaagt
  61 aacttaatat ccaacttgat ccacacttgt gcaagtttca atggttacca ctaccaatta
 121 acagattctc ttctattttc ggttttagga agagtttcga aactctatta atgacacaaa
 181 caactaatta agttatatgt ggaaattcca gctagaattt taggattttg gtggtaattt
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 361 tgcacactgt taccgagtgt atatatagag acatttgatc ctcgttgagc catcaaggtc
 421 taaaaccaaa tcaatccttc tttaatttac ttattcttag tataacgttc acacctttтc
 481 ttacacattt acaactatat ATGGAGTCGT CACGGAGCCT CGAGCACGTG CTCTCCATGC
 541 AAGGTGGCGA GGATGACGCG AGCTACGTCA AAAACTGTTA CGGTCCAGCG GCACGTTTAG
 601 CCTTGAGCAA ACCAATGCTG ACGACAGCCA TCAACTCCAT AAAGCTCACC GAAGGTTGCT
 661 CCTCTCACCT GAAAATAGCC GATTTAGGTT GCGCAATCGG AGACAACACG TTTTCCACGG
 721 TGGAAACGGT GGTTGAGGTG TTAGGGAAGA AGCTAGCCGT GATTGACGGT GGAACCGAGC
 781 CGGAGATGGA GTTCGAGGTC TTCTTCTCTG ACTTGTCTTC CAACGATTTC AACGCGTTGT
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1381 AGGATCGAAG TCATGGAACA AGGGAGGGGT GTGGATTGAA GGAGCAGAAA AAGAAGTCGT
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1501 GGAGATTGTG GTAGGAGGAG TGTTGTTTAT GCTGATGGGT GGTCGACCTT CTGGCTCCGT
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1801 TTGCGGCGGC GATTGATCGT TGTGGTGGTT TTAAGATAGA GAAAACGGAG AATCTGATAA
1861 TAGCTGACCA CATGAATGGT AAGCAGGAAG AGTTGATGAA AGATCCGGAT TCGTACGGTC
1921 GAGATAGGGC TAACTATGCT CAAGCCGGTT TAAAGCCGAT CGTTCAGGCC TATCTTGGTC
1981 CTGACCTAAC TCATAAGCTC TTCAAACGgt acgcagttag agcagctgcc gacaaagaaa
2041 ttctcaacaa ctgtttctat catatgatcg ctgtttcagc gttagggttt aatgttatcg
2101 atatatgtac ataacatgcg tagtattata aaaagtattt cgacgacgaa atggtttgat
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2581 tcctaaacaa gacaatgaaa gatctttctt tcttcttcct caaaaaatac tccctcgcct
2641 cgagAATGCG AGGACGAGGT CCACGTAGCG GCTTTGCTAG CTCTTGTGGT GGTGATGGCT
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2761 ACACTCCTTT CGGTGGAGGA TCTCCGAGAA CTCTAGAGGA GATGATACTT CAGCTAGAGG
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3121 cgttggtttc atgattgata atatgatatt caccggtgta gatttgaatg tacgaaacaa
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3301 ttattgtgta tgatactatg atcaatacta atatcaactt ctgtttgtct gccgttaagG
3361 TCAGCAAGGA ACGCGTTGAA TCAGTACCCA CGTTTCTCAC TGGACGGTAA GGACGCTATG
3421 TACCGGTCAT CCTTCCGGCG CCATCTTGGC ACCAGTGCTG ACATGACGAT ACAGGGTGGG
3481 CGGAGATCCC ACTGCGGGGA TCAACGGACA TCAAAGAGAT CGAGCCAGAT GAGCTTGGAG
3541 ACAAAACGCC TGCCACGGAC AGTGGCCGGA GAGAGTGTGG TATGGTGCAA GACGGGGGTT
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3601 GTTGCAAAGC TGATGGGTCT GGAGATGATA CCTGTTCCGG ACAAGGGAAA ATCGGGAAAA
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3721 GTTAATGGTC GGACCGGTCC GACGACTGAA GCTTCTTGCT CGTCTGGAGG GTTTAACATT
3781 ACTAGACCAA TCGGAGCAGT TGGGTCGCCT AGTCGCATGG GTGGATGGCC CACAGCCCGT
3841 CTCCCATAGa cttacgatgc aaaactagcc cattaaaaac cttcatgtta tctataaatt
3901 ttcattactg attgattcag taactttcta tgtttctcat aaatcttcta gactacataa
3961 ttaaggattt ccaaaccaag cacacacatt tttgtgactc aattaattct ctctgagcga
4021 gaaatcttag gaagcaggca aaaacacagt atacaagttc tgtctcaagc tcagattttg
4081 gtttcagaca gagttggatg ttcgatggac gagagtacac tgcgattcaa ttggaaaata
4141 cctaacaaac tttggttgcg aaaacccaag aaattgtgga tttgtgttgg atatatcaga
4201 cggaaactcc taggtccgac atacccatga tccttttgtt caatgcaatc ctcttctgtc
4261 tcaaaagcga agtcatggaa gccacctaat gatttacgac aaaagaattt aatgagacgt
4321 tcatagcaat gaagctcaaa caatgggcg
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Sequences of 5' leader, 3' trailer, and introns (when applicable) are printed in lowercase.

Protein sequence:

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DLGCAIGDNT FSTVETVVEV LGKKLAVIDG GTEPEMEFEV FFSDLSSNDF NALFRSLDEK
VNGSSRKYFA AGVPGSFYKR LFPKGELHVV VTMSALQWLS QVPEKVMEKG SKSWNKGGVW
IEGAEKEVVE AYAEQADKDL VEFLKCRKEE IVVGGVLFML MGGRPSGSVN QIGDPDSSLK
HPFTTLMDQA WQDLVDEGLI EEEKRDGFNI PVYFRTTEEI AAAIDRCGGF KIEKTENLII
ADHMNGKQEE LMKDPDSYGR DRANYAQAGL KPIVQAYLGP DLTHKLFKRM RGRGPRSGFA
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LMGLEMIPVP DKGKSGKDKL GTLLKRERLR RRERTLDVNG RTGPTTEASC SSGGFNITRP
IGAVGSPSRM GGWPTARLP*
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C. Publication in Plant Journal (2001)

Alex C. McCormac*, Andrea Fischer*, A. Madan Kumar, Dieter Söll and Matthew J. Terry (2001) **"Regulation of *HEMA1* expression by phytochrome and a plastid signal during de-etiolation in *Arabidopsis thaliana*."** The Plant Journal 25(5), 549-561
(* these authors contributed equally to this work.)

The Plant Journal (2001) **25**(5), 549–561

Regulation of *HEMA1* expression by phytochrome and a plastid signal during de-etiolation in *Arabidopsis thaliana*

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Summary

The synthesis of 5-aminolevulinic acid (ALA) is the rate-limiting step for the formation of all plant tetrapyrroles, including chlorophyll and heme, and regulation of ALA synthesis is therefore critical to plant development. Glutamyl-tRNA reductase (GluTR) is the first committed enzyme of this pathway and is encoded by a small family of nuclear *HEMA* genes. Here, we have used transgenic Arabidopsis (*Arabidopsis thaliana* L. Col) lines expressing chimeric *HEMA1* promoter:*gusA* fusion genes, combined with RNA gel blot analyses, to characterise the light-mediated regulation of the Arabidopsis *HEMA1* gene during de-etiolation. *HEMA1* was expressed strongly, but not exclusively, in photosynthetic tissues and was shown to be light regulated at the transcriptional level by the phytochrome family of photoreceptors acting in both the far-red high irradiance and low fluence response modes. The *HEMA2* gene, which is expressed only in roots of seedlings, was not light regulated. Analysis of truncated *HEMA1* promoter constructs demonstrated that a –199/+252 promoter fragment was sufficient to confer full light-responsiveness to *gusA* expression. This fragment contained GT-1/I-box and CCA-1 binding sites that are implicated as the light-responsive *cis* elements. Both the full-length and truncated *HEMA1* promoters required the presence of intact chloroplasts for full expression, consistent with previous indications that light and plastid factor signals converge to co-ordinately regulate expression of photosynthesis-related nuclear genes. These results provide the most comprehensive analysis to date of the light-regulation of a tetrapyrrole biosynthetic gene and support a direct link between regulation of *HEMA1* transcription and chlorophyll accumulation during seedling de-etiolation.

Keywords: chloroplast development, chlorophyll synthesis, glutamyl-tRNA reductase, aminolevulinic acid, light regulation.

Introduction

5-aminolevulinic acid (ALA) is the precursor of both chlorophyll, the primary light-harvesting pigment of higher plants, and heme, an essential constituent of proteins involved in the electron transport processes of photosynthesis and respiration (Beale and Weinstein, 1991; von Wettstein *et al.*, 1995). The heme branch of the pathway also leads to the synthesis of the phytochrome chromophore (Terry *et al.*, 1993). In plants, ALA is synthesised from glutamate via the C_5 pathway (Kumar *et al.*, 1996). This pathway is located in the plastid and consists of three enzymatic steps. The first step involves the activation of glutamate by glutamyl-tRNA synthetase. The product of this reaction, glutamyl-$tRNA^{Glu}$, can be used for either ALA or protein synthesis. The first committed step of ALA synthesis is catalyzed by glutamyl-tRNA reductase (GluTR), which reduces the activated glutamate to glutamate-1-semialdehyde (GSA). GSA is then converted to ALA by the enzyme glutamate-1-semialdehyde-2-1-aminotransferase (GSA-AT).

The formation of ALA is the rate-limiting step for tetrapyrrole synthesis and is the primary determinant of the rate of chlorophyll synthesis in light-grown plants (Beale and Castelfranco, 1974; Beale and Weinstein, 1991). As a consequence, ALA synthesis is tightly regulated

through the co-ordinated action of a wide range of signals (Kumar *et al.*, 1996). These include light, mediated by the phytochrome photoreceptor family (Huang *et al.*, 1989; Kasemir, 1983), the circadian clock (Kruse *et al.*, 1997), cytokinin (Masuda *et al.*, 1995) and the developmental status of the plastid (Kumar *et al.*, 1999). Because of its strategic position as the first committed step of the pathway, GluTR is strongly implicated as the limiting enzymatic step in ALA biosynthesis and is therefore likely to be the principal regulatory target. There is substantial evidence to support GluTR in this role. For example, in isolated cucumber plastids, stimulation of ALA synthesis by either benzyladenine or light correlated to an increase in GluTR activity with no change in the activities of glutamyl-tRNA synthetase and GSA-AM (Masuda *et al.*, 1995; Masuda *et al.*, 1996). Similarly, studies on circadian regulation of ALA synthesis in barley (Kruse *et al.*, 1997) and tobacco (Papenbrock *et al.*, 1999) showed that ALA synthesis correlated with GluTR mRNA abundance, but not with expression of *GSA*, the gene encoding GSA-AT.

In higher plants, GluTR is encoded by a small family of *HEMA* nuclear genes. Three *HEMA* cDNA clones have been isolated from barley (Bougri and Grimm, 1996; Tanaka *et al.*, 1997) while two have been identified in cucumber (Tanaka *et al.*, 1996) and soybean (Sangwan and O'Brian, 1999). In Arabidopsis, two *HEMA* genes have also been characterised (Ilag *et al.*, 1994; Kumar *et al.*, 1996). These have 83% identity at the amino acid level, but differ substantially in their patterns of expression. The Arabidopsis *HEMA1* transcript appears as the dominant form in leaves and has been detected in all parts of the plant (Ilag *et al.*, 1994). By contrast, *HEMA2* was found only in roots and flowers (Kumar *et al.*, 1996). We now need to address the question of how these genes are differentially regulated in order to meet the diverse demands of the developing plant for tetrapyrrole biosynthesis. This is particularly important during seedling de-etiolation as plastid biogenesis leads to a greatly increased demand for tetrapyrroles (primarily chlorophyll) that, if not correctly met, results in abnormal plastid development and compromises the photosynthetic capabilities of the plant. Inhibition of the tetrapyrrole pathway by inhibitors or antisense approaches severely impairs the normal development of plastids (e.g. Kumar and Söll, 2000), while deregulation of the pathway, for example by far-red light treatments (Barnes *et al.*, 1996), can result in lethal photooxidative damage (Reinbothe *et al.*, 1996). The regulation of this pathway must therefore be exquisitely controlled in order to permit optimal chloroplast development. In contrast, the phenotype of transgenic tobacco plants in which *cab* transcripts were reduced to almost undetectable levels using an antisense approach was not affected (Flachmann and Kühlbrandt, 1995). Thus tetrapyrroles have a vital role in plastid biogenesis and it is perhaps not surprising that they appear to be intricately linked to a signal indicating the developmental status of the plastid (Kropat *et al.*, 1997). Indeed, most of the *gun* (*genomes uncoupled*) mutants isolated in Arabidopsis (Susek *et al.*, 1993) are related to tetrapyrrole biosynthesis (J. Chory, personal communication).

The *HEMA1* gene should therefore be considered as a key component of the plastid developmental program and regulation of *HEMA1* expression is likely to play a critical role during chloroplast biogenesis. To date experiments on light regulation of *HEMA* genes have either looked at simple transitions to white light (Bougri and Grimm, 1996; Tanaka *et al.*, 1996) or, in the case of Arabidopsis, have been restricted to mature, light-grown plants (Ilag *et al.*, 1994). To understand the role of *HEMA* genes during the crucial period of de-etiolation we have focused on the light regulation of *HEMA1* expression in dark-grown seedlings exposed to a range of different light conditions. To do this we have created transgenic lines of Arabidopsis containing a series of chimeric *HEMA1* promoter:*gusA* (GUS) genes that have allowed us to compare *gusA* expression driven by a full-length *HEMA1* promoter with that produced by a series of truncated *HEMA1* promoters. In addition, we have also analyzed expression of an Arabidopsis *Lhcb1*2* promoter:*gusA* construct (Susek *et al.*, 1993) that is likely to reflect the requirement for chlorophyll but not for the other major tetrapyrrole product, heme.

Results

Analysis of the HEMA1 *promoter*

We determined the transcription start site for *HEMA1* using a 5′ RACE approach. This analysis yielded multiple mRNA products that terminated at different 5′ ends. Similar results were obtained by Ilag *et al.* (1994) using primer-extension analysis of *HEMA1* transcripts. The most distal end was mapped 252 nucleotides upstream of the translation start site (Figure 1a) and conforms to the consensus motif (PyAPyPy) for eukaryotic transcription start sites (Breathnach and Chambon, 1981). The most commonly represented 5′ ends were at positions +105 and +157 (numbered from the most distal start site) and 86% of clones (from a total of 51 sequenced) mapped at, or between, these two positions (shown as vertical arrows in Figure 1a). The distribution of mRNA 5′ ends was found to be the same whether the clones originated from the chimeric *HEMA1:gusA* or native *HEMA1* genes or if the products were from RNA pools isolated from mature green plants or seedlings (see Experimental procedures). These findings suggest that multiple transcription start sites are utilized to initiate *HEMA1* synthesis. This is consistent with the fact that no consensus TATA box is found 30 bp upstream of the most distal start site (Figure 1a).

Sequence analysis of the full-length *HEMA1* gene promoter (GenBank accession number AF295364) using the PLACE database (Higo *et al.*, 1998) revealed several GT1-binding sites, four of which included putative I-box/GATA core sequences (Figure 1a,b). The most proximal of these was ~30 bp upstream of the most distal transcription start site (Figure 1a). We also found a number of additional motifs that have been implicated in light-regulated gene expression (Figure 1a; see later for discussion).

HEMA1-*promoter driven expression of a* gusA *reporter gene*

A series of transgenic Arabidopsis lines were created in which upstream fragments of *HEMA1* were fused to the *gusA* coding region (Figure 1b). In order to include all potential regulatory elements in the upstream untranslated region, the protein coding region of *HEMA1* was replaced precisely with the *gusA* coding sequence at the ATG start codon, leaving the 5′ untranslated genomic region intact. The 3′ region, however, was replaced by the *nos* polyA signal. The *HEMA1* gene contains 2 introns that were also excluded in the promoter:*gusA* fusion construct.

For both the full-length *HEMA1* promoter and each promoter deletion construct, three independent homozygous lines were isolated which showed a 3 : 1 ratio of transgene segregation indicating a single locus insertion. The presence of a single locus was also confirmed by Southern blotting (data not shown). GUS activity was assayed in cotyledons from each of these three lines under continuous red (R) irradiation. GUS activity was similar whether driven by the full-length promoter or the promoter-deletion constructs with the exception of the –991/+252 and –199/+252 fragments that resulted in ~50% lower levels of reporter gene expression (Figure 1c). This difference in expression levels was also observed in the hypocotyl and roots (data not shown) and was consistently seen in plants grown under a variety of light conditions or in the dark (see later and data not shown). These results indicate the presence of non-specific enhancer (–708 to –199; –1355 to –991) and repressor (–991 to –708) elements within the *HEMA1* promoter. In the following analysis, quantitative data is shown from one line only of each deletion construct in order to minimise standard error calculations; qualitative patterns of responsiveness were, however, confirmed for at least two independent lines.

Localization of HEMA1 *expression*

We investigated the localization of *HEMA1* expression by histochemical GUS staining and *in situ* hybridization. Staining for GUS activity in the transgenic lines containing the full-length *HEMA1* promoter, using a histochemical substrate, showed localization of reporter expression

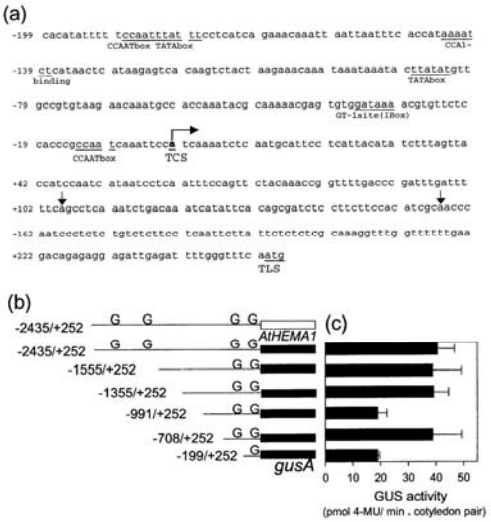

Figure 1. The *HEMA1* promoter and *gusA* reporter constructs used in this study.
(a) The proximal 451 bp of the Arabidopsis *HEMA1* promoter showing the translation (TLS) and primary transcription (TCS) start sites. The two vertical arrows at +105 and +157 indicate two additional sites commonly represented after sequencing of 5′ RACE clones. TATA boxes and potentially important regulatory *cis*-elements are also shown. (b) Promoter-deletion constructs of the 5′ untranslated region of *HEMA1* (up to the translation initiation codon) were placed upstream of the *gusA* coding sequence. The numbers correspond to nucleotides from the transcription start site and G indicates the position of a GT-1 binding site containing an I-box motif. (c) GUS activity driven by each *HEMA1* promoter fragment. Seedlings were grown for 3 d under continuous red light and the cotyledons were excised and assayed fluorometrically for GUS activity. Values shown are the mean (± SE) from three independent homozygous lines.

throughout both dark-grown (Figure 2a–d) and light-grown seedlings (Figure 2e–h). Comparisons of histochemical GUS localization with *in situ* hybridization for the native *HEMA1* transcript (Figure 2i,j) revealed very similar patterns. With both techniques, staining was seen strongly in the cotyledons of both etiolated and green seedlings. A characteristically patchy distribution of stain was observed during greening of cotyledons (Figure 2e,f,i), but no particular morphological structure could be associated with these foci. Staining was seen along the length of the hypocotyls in etiolated seedlings, but was more uneven in light-grown seedlings with strong local staining frequently found at the site of emergence of lateral root primordia. Strong staining was seen throughout the roots of both dark- and light-grown seedlings (Figure 2a,e,g) and this was most intense at the root cap (Figure 2c). The apical meristem was also strongly stained (Figure 2d). An identical pattern of stain distribution was observed for both the full-length *HEMA1* promoter and the –199/+252 construct

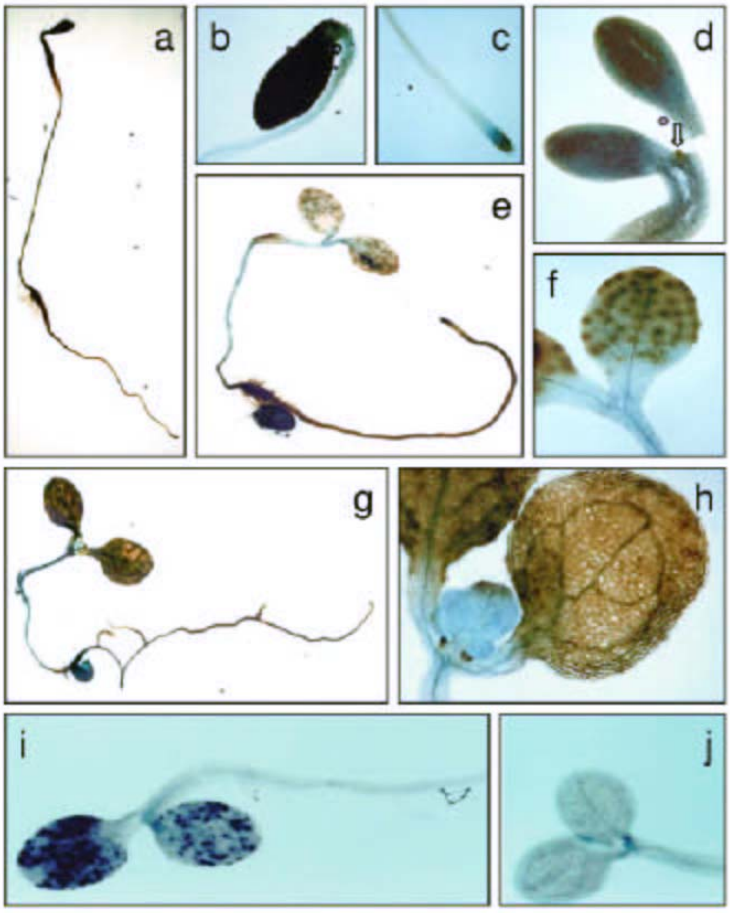

Figure 2. Localization of *HEMA1* expression in Arabidopsis.
(a–h) Histochemical localization of GUS activity expressed under the full-length (–2435/+252) *HEMA1* promoter. (a) etiolated seedling. (b–d) Details of etiolated seedlings following shortened staining periods showing (b) cotyledon (c) root tip and (d) apical meristem. (e) seedling exposed to white light (WL) for 2 days and (f) detail of cotyledon following a shortened staining period. (g) Seedling grown under WL for 1 week and (h) light-grown seedling showing expanded cotyledons and expanding first true leaf. (i–j) *In situ* hybridization for *HEMA1* transcripts in light-grown Arabidopsis seedlings using antisense (i) and sense (control) (j) RNA probes.

(data not shown) although at a lower overall expression level, consistent with the fluorimetric data (Figure 1c).

HEMA1 *expression is light regulated*

We examined the light regulation of *HEMA1* expression by RNA gel blot analysis. *HEMA1* mRNA could be detected at a low level in etiolated seedlings and was dramatically elevated following exposure to irradiation by various narrow- and broad-waveband light sources for 1 day (Figure 3a). Continuous irradiation by red (Rc), far-red (FRc) or blue (Bc) wavelengths each produced a strong induction of *HEMA1* expression similar to that under continuous white (Wc) (Figure 3a,b). Transcript accumulation from the *Lhcb* gene family displayed a very similar pattern of wavelength responsiveness (Figure 3a). In contrast to this strong light effect on *HEMA1*, hybridization with a *HEMA2*-specific probe revealed no significant change in the total abundance of *HEMA2* transcripts upon light treatment of etiolated seedlings (Figure 3c). This indicates that *HEMA1* is the primary *HEMA* gene involved in de-etiolation in Arabidopsis, a result that is completely consistent with the tissue-specific expression of these genes (Figure 2; Kumar *et al.*, 1996).

We also examined the light induction of *HEMA1* using the *HEMA1* promoter:*gusA* transgenic lines. Almost identical results were obtained to those observed by RNA gel blot analysis. GUS activity was detected in dark-grown tissues (Figure 4a), but levels were strongly elevated when etiolated seedlings were exposed to irradiation with Rc, FRc, Bc and Wc for 3 days (Figure 4a). The increased expression under FRc is indicative of a typical high irradiance response (FR-HIR). Relative responsiveness to the Rc, FRc, Bc and Wc light-sources was essentially the

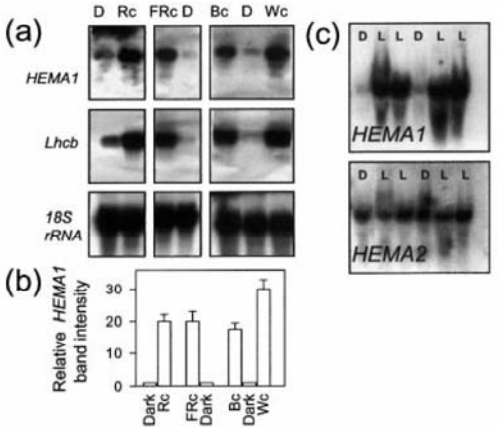

Figure 3. Light regulation of *HEMA1* mRNA abundance.
(a) RNA gel blots showing *HEMA1* and *Lhcb* mRNA accumulation under continuous irradiation by red (Rc), far-red (FRc) or blue (Bc) light sources or in the dark (D). Etiolated Arabidopsis seedlings were irradiated for 1 day and total RNA was isolated from whole seedlings. (b) Densitometric quantification of *HEMA1* transcript levels showing the mean (± SE; $n = 3$) relative increase over corresponding D control seedlings after correction for *18S* rRNA levels. (c) RNA gel blot of total RNA from seedlings grown under Wc (L) or in the D. Hybridization was performed sequentially on the same blot with gene-specific probes for *HEMA1* and *HEMA2* transcripts.

same for the full-length *HEMA1* promoter and each of the truncated promoter fragments (Figure 4a). In control experiments, these light treatments also resulted in the same relative induction of the *Lhcb1*2* promoter:*gusA* construct (Figure 4a). The light-mediated effect on *HEMA1* promoter activity was maximal in the cotyledons, but was also significant in the hypocotyls (Figure 4b). The roots showed little or no response (Figure 4b).

The above results demonstrate that the activity levels of the GUS enzymatic reporter, as directed by the *HEMA1* promoter, resembled the qualitative light response of native *HEMA1* transcript accumulation. To further investigate how closely regulation of the reporter gene correlated with that of the intact *HEMA1* gene, *gusA* expression was also assayed at the transcript level by RNA gel blot analysis. The time-course of mRNA accumulation directed from the *HEMA1* promoter was assayed in etiolated seedlings transferred to Rc. A rapid elevation within 2 h from the start of irradiation was observed for both native *HEMA1* and *gusA* transcript levels (Figure 5a,b). Under continuous irradiation levels continued to increase for at least the initial 24 h period, although at a substantially slower rate. The further increase in mRNA levels seen at the 48 h time-point may reflect progressive seedling development, i.e. the early development of the secondary

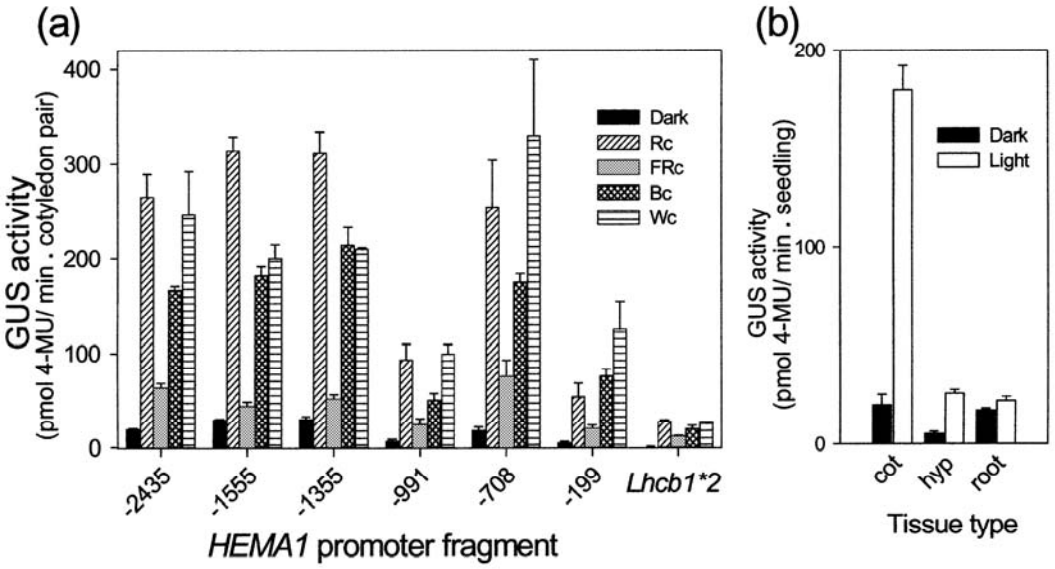

Figure 4. Light regulation of *HEMA1* promoter:*gusA* expression.
(a) GUS activity driven by *HEMA1* promoter constructs (see Figure 1b) or the *Lhcb1*2* promoter following continuous irradiation by red (Rc), far-red (FRc), blue (B) and white (Wc) light sources. Etiolated seedlings were grown for 3 days in the dark (D) and exposed to light treatments for 3 days. Data shown are the mean ± SE of $n = 3$ independent experiments, each of which sampled a minimum of 30 seedlings. The total protein content (μg.cotyledon pair^{-1}) under the different light conditions was as follows (mean ± SE): D, 0.23 ± 0.03; Rc, 0.41 ± 0.06; FRc, 0.31 ± 0.04; Bc, 0.40 ± 0.03; Wc, 0.55 ± 0.03. (b) The effect of Rc on *HEMA1*-driven GUS activity in cotyledons (cot), hypocotyls (hyp) and roots (mean ± SE; $n = 3$).

 The Plant Journal, (2001), **25,** 549–561

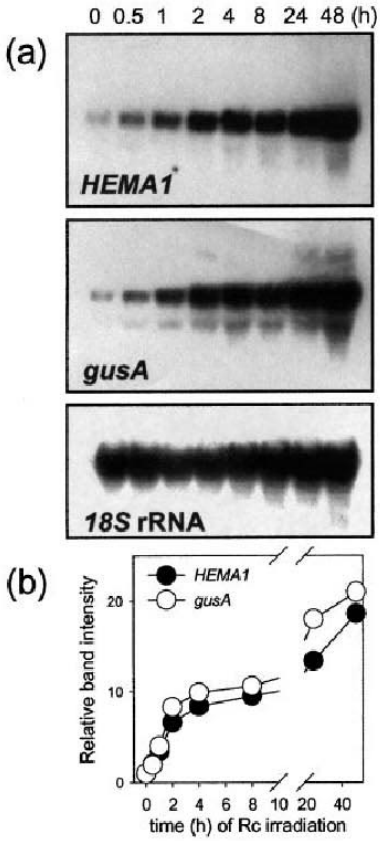

Figure 5. Kinetics of *HEMA1* expression under continuous red light.
(a) RNA gel blot analysis of native *HEMA1* and *gusA* mRNA accumulation following irradiation with continuous red light (Rc) for various time periods. The same RNA blot was probed sequentially with *HEMA1* and *gusA* transcripts. (b) Densitometric quantification of relative band intensities of *HEMA1* and *gusA* transcripts after correction for *18S* rRNA levels plotted against irradiation period.

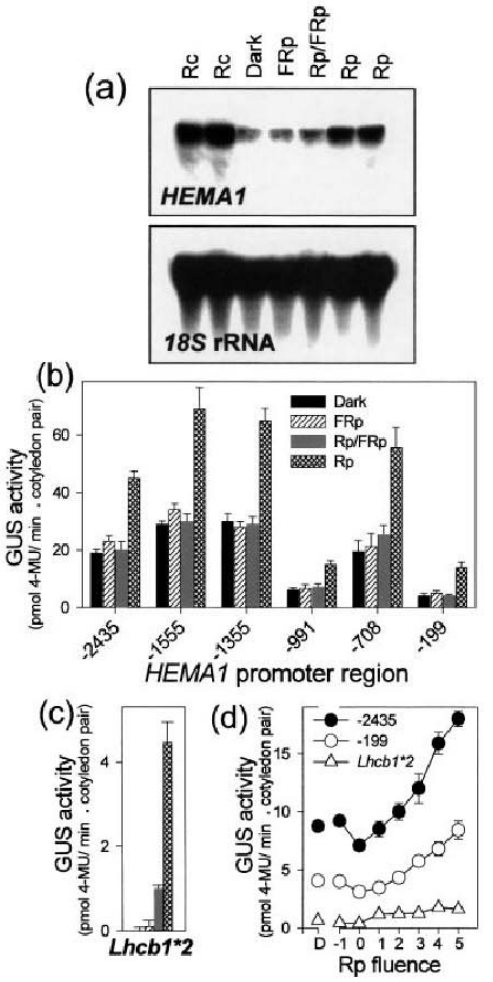

Figure 6. Photoreversibility of light-induced *HEMA1* expression.
(a) RNA gel blots showing *HEMA1* mRNA accumulation after saturating red (Rp), far-red (FRp) or Rp/FRp light pulses or under continuous red (Rc) irradiation. Total RNA was extracted from etiolated seedlings 2 h after each light pulse treatment. (b–c) Photoreversibility of light-induced *HEMA1* promoter:*gusA* expression. GUS activity, driven by *HEMA1* promoter constructs (b; see Figure 1b) or the *Lhcb1*2* promoter (c) after saturating red (Rp), far-red (FRp) or Rp/FRp light pulses. Etiolated seedlings were grown for 3 days in the dark (D) and exposed to 4 light pulses/day for 3 days. Data shown are the mean ± SE of $n = 3$ independent experiments, each of which sampled a minimum of 30 seedlings. (d) Fluence-response relationship of an Rp treatment. GUS activity driven by the full-length (– 2435/+252), the most proximal 451 bp *HEMA1* promoter (– 199/+252) or the *Lhcb1*2* promoter was measured following 4 Rp d^{-1} of different fluences for 3 days.

leaves. Thus, light-induced changes in mRNA levels of the *gusA* and *HEMA1* genes were observed to follow very similar time-courses with the relative magnitude of the maximal response (~10-fold D levels) comparable for each transcript (Figure 5b).

Phytochrome mediates the light regulation of HEMA1 *expression*

Expression under Rc and FRc is indicative of responses mediated by the phytochrome family of photoreceptors. To examine the R/FR reversibility of *HEMA1* transcript accumulation we measured *HEMA1* expression following light pulse treatments. Etiolated seedlings of Arabidopsis were subjected to a 15 min Rp, returned to darkness and total RNA was extracted after 2 h and probed for the *HEMA1* transcript. Figure 6 shows that a single Rp resulted

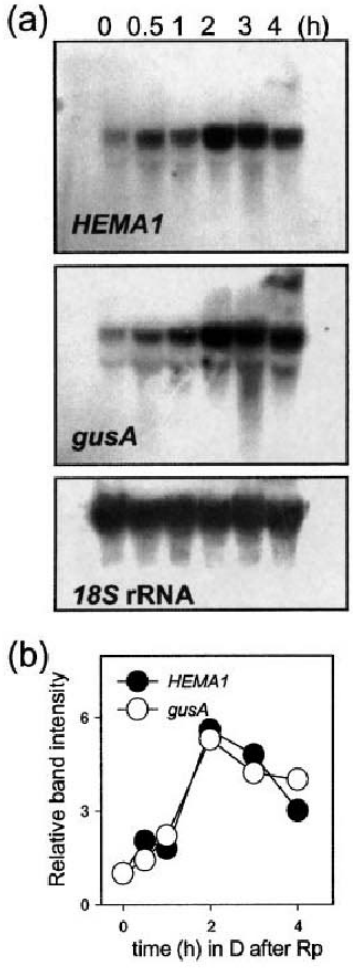

Figure 7. Kinetics of *HEMA1* expression following a red light pulse.
(a) RNA gel blot analysis of native *HEMA1* and *gusA* mRNA accumulation in the dark (D) following irradiation with a 15 min red light pulse (Rp). The same RNA blot was probed, sequentially, with *HEMA1* and *gusA* transcripts. (b) Densitometric quantification of relative band intensities of *HEMA1* and *gusA* transcripts after correction for *18S* rRNA levels plotted against time following an Rp.

in significant induction of *HEMA1* expression compared to seedlings maintained in D (Figure 6a). This response to a single Rp was comparable to that under continuous R (Figure 6a). The increase in *HEMA1* transcript levels in response to a single saturating Rp could be reversed by the immediate delivery of a saturating FRp (Figure 6a) consistent with the low fluence (LF) mode of phytochrome-mediated response. An FRp delivered alone did not produce any detectable increase in *HEMA1* mRNA, relative to the dark control seedlings.

We also examined the effect of light pulses on *HEMA1* promoter:*gusA* expression. A single saturating Rp delivered did not produce a measurable increase in GUS activity relative to D control seedlings (data not shown). However, the delivery of repeated pulses (= 12 pulses/d for 3 days) was able to fully replicate the induction by Rc and a delivery rate of 4 pulses/d produced an intermediate response (data not shown). Whereas Rc and Rp (12/d) regimes resulted in expansion and greening of the etiolated cotyledons, Rp (4/d) over a 3 day period did not produce such gross changes in seedling morphology and this regime was therefore adopted for further experiments.

Elevation of GUS activity in response to the 4 pulses/d regime was detected only in the cotyledons (Figure 6b) with no induction apparent in the hypocotyls and roots (data not shown). The induction by Rp (4/d) could be completely abolished by the subsequent delivery of an FRp immediately following each Rp (Figure 6b). FRp (4/d) alone induced no effect on GUS activity relative to D seedlings. This same pattern of R/FR-reversibility was displayed by each of the *HEMA1* promoter-deletion constructs (Figure 6b). The *Lhcb1*2* promoter:*gusA* reporter was similarly responsive to Rp deliveries, but in this case the response was not fully reversible by FR and a FRp alone mediated a small induction of GUS activity relative to D (Figure 6c). Responsiveness to FRp is interpreted as a phytochrome-mediated very low fluence response (VLFR) that is saturated by <3% Pfr/Ptot.

The *HEMA1* promoter-mediated response of GUS activity to Rp was used to determine the fluence-response relationship (Figure 6d). GUS activity increased over the fluence range 10^2–10^5 μmol m^{-2}, consistent with previously characterized LF responses. The −199/+252 promoter fragment demonstrated a comparable fluence relationship providing further evidence that the reductions in GUS activity for this construct were through a general enhancer effect and that this region is sufficient for full light responsiveness. No VLFR mode of activation at R fluences $<10^1$ μmol m^{-2} was observed, consistent with the failure of a FRp to induce *HEMA1*-transcript (Figure 6a) or GUS-activity levels (Figure 6b). *Lhcb1*2* promoter activity, however, did demonstrate small activity increases within this range (Figure 6d). We also noted a small, but consistent, decrease in *HEMA1*-driven GUS activity in response to a Rp with a fluence of ~1 μmol m^{-2}. This feature of the response curve is similar to that seen previously for anthocyanin synthesis in tomato under R (Kerckhoffs *et al.*, 1997).

As the detection of elevated GUS activity required the delivery of repeated Rp, we wished to confirm that this was a genuine LFR and the introduced *HEMA1* promoter could, indeed, respond to a single Rp. Therefore, etiolated seedlings of a transgenic Arabidopsis line containing the full-length *HEMA1* promoter:*gusA* reporter gene were

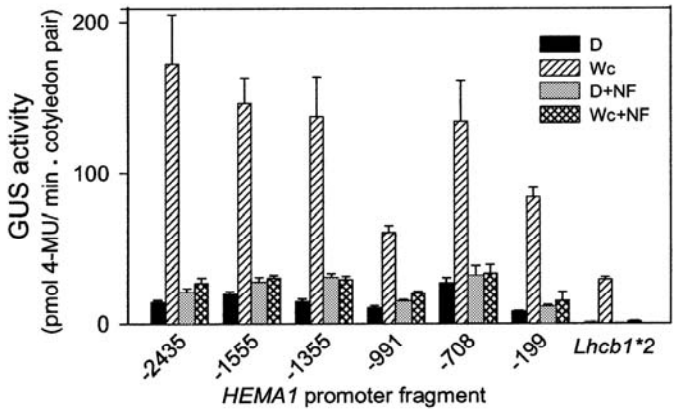

Figure 8. Regulation of *HEMA1* promoter:*gusA* expression by a plastid signal.
The effect of Norflurazon (NF) on GUS activity driven by *HEMA1* promoter constructs (see Figure 1b) or the *Lhcb1*2* promoter following a continuous white light (Wc) treatment. Etiolated seedlings were grown for 3 days in the dark (D) in the absence or presence of 5 μM NF and transferred to Wc for a further 3 days. Data shown are the mean ± SE of n≥3 independent experiments each of which sampled a minimum of 30 seedlings.

subjected to a single 15 min Rp, returned to darkness and total RNA extracted at various intervals. The time-dependent accumulation of transcripts from the native *HEMA1* gene and the *gusA* reporter gene were each followed by successive hybridizations to the blotted RNA samples (Figure 7a). The abundance of both transcripts increased rapidly and substantially in response to a single Rp reaching, in each case, a maximum of approximately sixfold D levels approximately 2 h following the Rp (Figure 7b). Levels of *HEMA1* mRNA declined back to those of the dark control seedlings within 24 h of the light pulse. Transcript levels of *gusA* also declined following the peak at 2 h but persisted after 24 h at approximately 30% of peak levels (data not shown). This result demonstrates that the transgene is faithfully mirroring *HEMA1* promoter activity within the Arabidopsis host. The failure of this transcriptional response to be observed as a significant increase in GUS activity was probably due to the difficulty of detecting a transient rise within an already substantial background of dark expression.

Plastid factor-dependence of the HEMA1 *promoter maps with light responsiveness*

It has already been established, through RNA gel blot assays for the native transcript that light-mediated induction of the *HEMA1* gene is dependent on the presence of intact chloroplasts (Kumar *et al.*, 1999). Treatment of seedlings with the phytoene desaturase-inhibitor herbicide, Norflurazon, under WL leads to photobleaching of the chloroplasts and the loss of light-induced expression of photosynthesis-related genes. Since phytochrome synthesis is unaffected under these conditions (Thomsen *et al.*, 1993), this has been interpreted as resulting from the absence of a plastid signal required for normal gene expression. As seen for native transcription, light-responsive accumulation of GUS activity from the *HEMA1* promoter was severely inhibited by Norflurazon application (Figure 8). As expected, this effect was most pronounced in the cotyledons although an inhibitory effect of Norflurazon in the light was also observed in hypocotyls (data not shown). The effect of photobleaching on *HEMA1* expression was identical for the full-length *HEMA1* promoter and each of the promoter deletions, including the –199/+252 region (Figure 8).

Discussion

Transcriptional regulation of HEMA1 *by light*

The *HEMA1* gene has previously been shown to be strongly expressed in mature light-grown plants and down-regulated upon dark-adaptation of these plants (Ilag *et al.*, 1994). Here, we have characterized the light-regulation of *HEMA1* expression in seedlings undergoing the transition from etiolated to autotrophic growth using two different approaches: direct measurement of steady state mRNA abundance by RNA blot analysis and quantitation of *HEMA1* promoter activity using a GUS reporter assay. Using both methods, *HEMA1* expression was detected in etiolated tissues and was increased significantly under Rc, FRc, Bc and Wc and also by an Rp regime that induced expression in the absence of major changes in seedling morphogenesis. In contrast, *HEMA2* was not light regulated. *HEMA1* was also highly expressed in photosynthetic tissues with expression dependent on the presence of functional plastids. These features are all characteristic of the general expression patterns of photosynthesis-related genes (Batschauer *et al.*, 1994; Taylor, 1989; Terzaghi and Cashmore, 1995) and are consistent

with the role of *HEMA1* in encoding a key regulatory enzyme in chlorophyll biosynthesis.

Previous studies on the light regulation of *HEMA* genes have largely been restricted to establishing a simple white light requirement for maximal expression (Bougri and Grimm, 1996; Ilag *et al.*, 1994; Tanaka *et al.*, 1996). These studies utilized continuous WL treatments that would also have resulted in gross developmental changes and did not demonstrate a direct role for individual photoreceptors in the observed light response. The results shown in the present study implicate a number of different photoreceptors in the light regulation of *HEMA1*, including the phytochrome photoreceptor family acting in two different response modes. The response to FRc is characteristic of an FR-HIR, a response that is well established as being mediated by phytochrome A (Whitelam and Devlin, 1997). Similarly, the Rp/FRp reversible LFR has been attributed primarily to phytochrome B in Arabidopsis (Whitelam and Devlin, 1997). Further experiments using phytochrome-deficient mutants will be required to confirm these designations. We failed to detect *HEMA1* induction through the VLF phytochrome response mode. However, this may be attributed to the genetic background used for these studies, as the Columbia ecotype is generally impaired in this particular response (Yanovsky *et al.*, 1997). The relatively strong response to Bc also suggests that the cryptochromes may play an important role in the light regulation of *HEMA1*, though again this will need to be confirmed by mutant analysis.

Expression of the *HEMA1* promoter:*gusA* reporter gene to the different light regimes faithfully mirrored that of the native *HEMA1* gene. This includes the similar accumulation kinetics of the native *HEMA1* and *gusA* mRNAs observed here in response to either Rc or a single Rp and the similar magnitude of light induction under all the light regimes examined. These results indicate that the difference in coding sequences of the two transcripts did not exert a significant effect on their light-induced expression and from this we infer that regulation of *HEMA1* mRNA abundance is controlled exclusively by the upstream, non-coding sequences. This suggests that significant transcript stability effects are unlikely and that transcriptional control is the primary regulator of *HEMA1* expression, as is generally (though not exclusively) the case for the control of light-regulated genes (Batschauer *et al.*, 1994; Terzaghi and Cashmore, 1995). It should be noted though that the promoter:*gusA* constructs contained the first 252 nucleotides of non-coding sequence from the *HEMA1* gene and some regulation through transcript stability cannot be completely discounted. There was an indication that the *HEMA1* transcript was less stable than *gusA* in D following an Rp. However, this effect was relatively minor and both transcript levels peaked 2 h after the Rp.

The –199/+252 region of the HEMA1 *promoter can confer light and plastid-factor regulation*

Analysis of *HEMA1* promoter deletion constructs demonstrated that the –199/+252 region could respond to light treatments in a qualitatively identical manner to the full-length promoter (–2435/+252). It is a consistent feature of light-regulated promoters that all the required *cis* elements for light-dependent transcriptional regulation are located in a short region immediately 5′ to the transcription start site. For example, Kusnetsov *et al.* (1996) analyzed truncated promoters of seven nuclear-encoded, photosynthesis-related genes from spinach for their ability to respond to a white light treatment. For all seven promoters, regions ranging between –130/+60 (*PetE*) and –62/+173 (*AtpC*) were sufficient to confer light responsiveness. Indeed, a 146-bp fragment from the tobacco *Lhcb1*2* promoter was shown to confer the full-range of VLFR, LFR and HIR phytochrome responses to a truncated CaMV*35S* promoter (Cerdán *et al.*, 1997). The –199/+252 *HEMA1* promoter fragment was also able to respond to phytochrome acting in both LFR and HIR modes.

A number of different light-responsive elements (LREs) have been identified in promoters of light-responsive genes (Terzaghi and Cashmore, 1995) and these have been shown to act in a combinatorial manner to confer light regulation (Argüello-Astorga and Herrera-Estrella, 1996; Puente *et al.*, 1996). In our study, no G-box elements were found within the *HEMA1* promoter, but other consensus sequence motifs were identified including several for GT1-binding sites. GT1-elements are found within the promoters of a wide variety of genes, not all of which are light regulated. However, when associated with an I-box or I-box-related motif (known also as a GATA motif) they appear to be critical components of light-responsive promoters (Argüello-Astorga and Herrera-Estrella, 1996; Puente *et al.*, 1996). Indeed, GT-1 elements placed in tandem with GATA motifs can confer regulation by Wc, Bc, Rc and FRc to a minimal *NOS101* promoter (Chattopadhyay *et al.*, 1998) and it has been proposed that phytochrome regulation through this *cis*-element is mediated by the CGF-1 binding factor (Anderson *et al.*, 1997). The *HEMA1* promoter contains putative I-box core motifs as an integral part of five GT-1 binding sites with one of these elements strategically placed within the –199/+252 promoter fragment close to the transcription start site. The promoter region of the *HEMA2* gene also contains GT1-binding site homologies and I-box motifs but, unlike *HEMA1*, these are not associated as a single unit within the vicinity of the transcription start sites. In addition to the GT-1/I-box motifs, the –199/+252 *HEMA1* promoter fragment contains two other consensus motifs that have been implicated in light regulation. These include two CCAAT boxes and an extended sequence

related to an ABF-2 binding domain (Argüello-Astorga and Herrera-Estrella, 1996) that contains the AAAATCT motif that is predicted to bind the Myb-related factor, CCA1 (Wang *et al.*, 1997). Together with I-box motifs these elements have been shown to be part of a conserved modular arrangement found in the promoters of the *Lhcb* family, including *Lhcb1*2* (Argüello-Astorga and Herrera-Estrella, 1996). These observations suggest a molecular basis for the co-ordinated synthesis of chlorophyll and chlorophyll-binding proteins.

The −199/+252 *HEMA1* promoter also contained all of the information required for correct tissue-specific expression and the ability to respond to the loss of a plastid factor. This type of convergence has been noted previously. Kusnetsov *et al.* (1996) found a strict correlation between responses to light and a plastid signal for 32 different promoter deletion constructs from seven different photosynthesis-related genes. These results demonstrate that there is a significant interaction between these signaling pathways. One possible hypothesis that has been suggested is that light regulation of gene expression is actually mediated through changes in plastid development and the subsequent production of a plastid signal (López-Juez *et al.*, 1996). This signal could also convey the required tissue specificity leading to the light-induced expression of photosynthesis-related genes only in photosynthetic tissues. It should be noted however, that the −199/+252 *HEMA1* promoter fragment retained the ability to respond to not only these signals, but also to factors that confer expression in non-photosynthetic tissues.

The significance of HEMA1 *regulation for plastid development*

The expression pattern of *HEMA1* was indicative of the dual role for GluTR in meeting the demand for both heme (required by all cells) and chlorophyll. Thus, *HEMA1* was expressed strongly in photosynthetic tissues, such as cotyledons and primary leaves, where there is a high demand for both chlorophyll and heme, and also in hypocotyls and roots for which heme is the major tetrapyrrole product. The intense GUS staining seen at the sites of root and shoot primordia indicates a correlation with cell division events and therefore also a strong requirement for heme. In contrast to *HEMA1*, *HEMA2* expression is confined to roots and flowers (Kumar *et al.*, 1996) suggesting that it is involved exclusively in heme synthesis. This interpretation is supported by the data on light regulation. *HEMA2* was not light regulated, while *HEMA1* was strongly light induced in photosynthetic tissues with little or no induction in hypocotyls and roots, respectively. A similar pattern of expression was seen for *hemA1* and *hemA2* in cucumber (Tanaka *et al.*, 1996), while in barley, *hemA1* and *hemA2* meet the tetrapyrrole requirement of photosynthetic tissues with *hemA3* expressed primarily in roots (Bougri and Grimm, 1996; Tanaka *et al.*, 1997). As discussed above the light-responsiveness of these genes correlates well with the chloroplast content of a particular tissue and chloroplast signals would provide a suitable mechanism for tailoring the requirement for *HEMA1* expression to the demand for chlorophyll. Chloroplast signals would also serve to co-ordinate *HEMA1* with *Lhcb* expression in these tissues and can be thought of as 'gating' light-induction of gene expression – a similar phenomenon perhaps to that proposed for the circadian clock (Millar and Kay, 1996).

It is well established that ALA synthesis is limiting for chlorophyll synthesis (Beale and Weinstein, 1991) and that light stimulates the rate of ALA synthesis through phytochrome signaling (Huang *et al.*, 1989; Kasemir, 1983). The demonstration of a rapid (within 2 h) and large (10-fold induction over D levels) rise in *HEMA1* expression in this study is compatible with the role of the gene product (GluTR) in effecting maximal ALA synthesis during greening. The time-course for accumulation of *HEMA1* mRNA in etiolated seedlings exposed to continuous irradiation fits well with that reported for the kinetics of chlorophyll accumulation during the greening period (e.g. Lifschitz *et al.*, 1990), for which there is an initial lag-phase of 2 h followed by increasing rates of chlorophyll synthesis in the initial 12 h period. The response kinetics of *HEMA1* transcript abundance following a single Rp, and the fluence response characteristics of promoter activity also correlate well with those reported for the potentiating effect of a Rp on the greening response (Ken-Dror and Horowitz, 1990; McCormac, 1993), consistent with the proposal that transcriptional expression of *HEMA1* is the main contributor to this effect. In contrast, *GSA1*, encoding the second committed enzyme of ALA synthesis, showed only a moderate light-dependent increase in mRNA abundance (Ilag *et al.*, 1994; A.C. McCormac and M.J. Terry, unpublished results). As GSA-AT levels are also unresponsive to a plastid signal (Kumar *et al.*, 1999), it is clear that regulation of *HEMA1* is the primary determinant of the rate of ALA and therefore chlorophyll synthesis in Arabidopsis. In this context it is interesting that *HEMA1* is upregulated under FRc. Continuous FR has been shown to result in changes in plastid development that are lethal following transfer to WL (Barnes *et al.*, 1996). An increase in *HEMA1* expression would be predicted to result in an increase in potentially phototoxic tetrapyrroles and it will be interesting to determine whether induction of *HEMA1* is important in this response.

Recent evidence has implicated tetrapyrroles as being important in plastid signaling. This includes the observations that Mg-protoporphyrin IX can induce two nuclear heat-shock genes in Chlamydomonas (Kropat *et al.*, 1997) and that most of the *gun* mutations in Arabidopsis (Susek

et al., 1993) are related to defects in tetrapyrrole metabolism (J. Chory, personal communication). As *HEMA1* is the key target for light regulation of tetrapyrrole synthesis and is itself regulated by a plastid signal, it has the potential to play a crucial role in the interaction between light and plastid-signaling during the critical period of de-etiolation and a precise understanding of *HEMA1* regulation will be important in the construction of models of plastid/nuclear signaling. Future work will focus on uncovering the nature of this role and elucidating the relationship between tetrapyrrole synthesis and the light regulation of plastid development.

Experimental procedures

Construction of vectors and plant transformation

An *Nco*I restriction site was introduced into the genomic clone of *HEMA1* (Ilag *et al.*, 1994) at the 5′ end of the open reading frame. Using *Nco*I and *Pst*I the entire region of the *HEMA1* gene downstream of the translation start site was removed and inserted into the *Nco*I–*Pst*I fragment of pRTL2-GUS (Restrepo *et al.*, 1990). Deletions of the 5′ upstream *HEMA1* sequence were generated by exonuclease III/S1 nuclease digestion and selected by size (see Figure 1). The sequence of the 5′ upstream regions and the size of the selected deletions were subsequently determined.

The six constructs were introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation. Wild-type Arabidopsis (*Arabidopsis thaliana* L.) plants (ecotype Columbia) were transformed by vacuum-infiltration (Bechtold *et al.*, 1993) and transformants were selected on hygromycin. Arabidopsis plants (ecotype Bensheim) expressing the *Lhcb1*2(CAB3/CAB180):gusA* reporter gene have been described previously (line pOCA108; Li *et al.*, 1995).

Light sources

Broad-band white light (WL) was provided by white fluorescent tubes (400 nm-700 nm = 130 $\mu mol/m^2\ s^{-1}$). Narrow waveband sources were provided by LED displays in environmental control chambers (Percival Scientific Inc., Boone, IA, USA). Red light (R) corresponded to a peak at 669 nm (25 nm band-width at 50% of peak magnitude) with a fluence rate of 80 $\mu mol/m^2\ s^{-1}$. Far-red light (FR) from the LEDs had a peak at 739 nm (25 nm band-width at 50% of peak magnitude) and was passed through two filters (#116 and #172; Lee Filters, Andover, UK) to remove $\lambda < 700$ nm to give a final fluence rate of 10 $\mu mol/m^2\ s^{-1}$. Blue light (B) had a peak at 470 nm (25 nm band-width at 50% of peak magnitude) and a fluence rate of 20 $\mu mol/m^2\ s^{-1}$.

RNA gel blot analysis

Total RNA was isolated from 3- to 5-days-old Arabidopsis grown aseptically on medium containing MS salts (Murashige and Skoog, 1962) and 1% (w/v) agar. Seedlings were frozen in liquid N_2 and ground in extraction buffer (phenol: 0.1 M LiCl, 0.1 M Tris/HCl pH 8.0, 10 mM EDTA and 1% (w/v) (SDS) (1 : 1, v/v). The resulting extract was partitioned with chloroform and RNA was precipitated in an equal volume of 4 M LiCl, followed by precipitation with ethanol. Total RNA (30 $\mu g\ lane^{-1}$ except for Figure 3c where 60 $\mu g\ lane^{-1}$ was used) was heat-denatured (65°C for 10 min) in the presence of 50% (w/v) formamide, separated on a denaturing 1.4% agarose gel (Sambrook *et al.*, 1989) and blotted onto Hybond-N (Amersham Pharmacia Biotech, UK). Equal loadings of RNA were calculated from ethidium bromide staining of samples prior to gel loading and confirmed by hybridization of the stripped blots with a flax *18S* rDNA fragment. Prehybridization and hybridization were performed in the presence of 50% formamide at 42°C (Sambrook *et al.*, 1989). Washings were carried out to a final stringency of 0.2 × SSC + 0.1% (w/v) SDS at 42°C. Probes were labeled with [α-^{32}P] dCTP using random hexanucleotide priming (Rediprime; Amersham Pharmacia Biotech, UK). *HEMA1* and *HEMA2* gene-specific probes were isolated as 3′ cDNA fragments by digestion of the cDNA clones (Kumar *et al.*, 1996) with *Sph*I + *Sal*I and *Xho*I + *Not*I, respectively. The *Lhcb* probe was isolated as a *Bam*H1 + *Sac*I restriction fragment and contained the majority of the coding region of the *Lhcb1*2* gene, although it is predicted to cross hybridize with other members of the *Lhcb* gene family. Blots were exposed onto X-ray film (Kodak BioMax MS, Amersham Pharmacia Biotech, UK) and densitometric scans of the resulting bands were performed using a digital imaging system (Alpha Innotech Corp., San Leandro, CA, USA) and the AlphEase software package.

5′ RACE analysis

Plants expressing the *HEMA1* promoter:*gusA* transgene were grown under two alternative light regimes and total RNA was isolated as described for RNA gel blot analysis. The first RNA pool was isolated from mature green plants, grown under Wc for 2–3 weeks. The second extraction was from etiolated seedlings (grown for 2 days in D), exposed to a saturating Rp and incubated in D for a further 3 h prior to RNA isolation. RNA was either used directly for cDNA synthesis in the case of etiolated seedling extracts, or following polyA mRNA purification with PolyATract (Promega Ltd, Southampton, UK) in the case of mature light-grown plants. First strand cDNA synthesis reactions were performed according to the SMART RACE cDNA Amplification Kit (Clontech Laboratories Inc., Paolo Alto, CA, USA) using Superscript II MMLV reverse transcriptase (Life Technologies Inc., Rockville, MD, USA) and gene-specific primers for *HEMA1* (5′-GCAAGCTTCTCACGCATCTCAACAGGAGC-3′) and *gusA* (5′-CGCAGCACGATACGCTGGCCTGCC-3′). For each RNA/primer combination, the PCR products were observed to migrate on a 1.5% agarose gel as a single band of approximately 500 bp (*HEMA1* primer) or 400 bp (*gusA* primer). Randomly selected clones of each of these products were sequenced at the 5′ mRNA ends. Clones for *HEMA1* were confirmed as originating from the mRNA template, as opposed to genomic DNA, by verifying that the first intron sequence had been correctly spliced.

In situ *hybridization*

Whole-mount *in situ* hybridization was performed as described by de Almeida Engler *et al.* (1994) with a 2% driselase (Sigma Chemical Co., St. Louis, USA) digestion step added to the prehybridization treatment. RNA probes were prepared *in vitro* using digoxigenin RNA labeling mix (Boehringer Mannheim GmbH, Mannheim, Germany) according to the manufacturer's protocol. Riboprobes were digested to an average length of 150 nucleotides by controlled alkaline hydrolysis (Cox *et al.*, 1984) and used at a final concentration of 3 $\mu g\ ml^{-1}$.

GUS histochemical analysis

Seedlings were vacuum infiltrated, for 10 min, in 100 mM sodium phosphate, pH 7.0, 0.1% formaldehyde and 0.1% (v/v) Triton X-100 followed by three washes in 50 mM sodium phosphate buffer, pH 7.0. Samples were then incubated for 8–24 h, at 37°C, in the histochemical stain solution consisting of 50 mM sodium phosphate buffer, pH 7.0, 1 mM EDTA, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide and 1 mg ml^{-1} 5-bromo-4-chloro-3-indolyl-β-D-glucuronide. Sequential washes in 25%, 50%, 75% and 90% (v/v) ethanol were used to dehydrate the tissues and remove chlorophyll. Photographs of stained seedlings were taken using an Olympus BH-2 camera $light^{-1}$ microscope facility (Olympus Optical Co. (UK) Ltd, London).

GUS fluorometric analysis

Quantitative assays of GUS activity were conducted by measuring 4-methyl umbelliferone (4-MU) production from the 4-methyl umbelliferyl glucuronide (MUG) substrate in a time-course assay (Jefferson *et al.*, 1987). Fluorescence was measured using the TD-360 fluorometer (GRI Ltd, Braintree, UK), which was calibrated using standard solutions of 4-MU. For each value, a minimum of 30 seedlings was sampled. Seedlings were dissected, at the time of harvest, by an excision just below the apex to isolate the cotyledons as a pair. Where separate measurements are shown for hypocotyls and roots, a second excision was made in the region of the transition zone of these two organs. Tissues were stored in extraction buffer (100 mM sodium phosphate, pH 7.0, 10 mM EDTA, 0.1% (v/v) Triton X-100, 0.1% (w/v) Sarkosyl and 10 mM,-mercaptoethanol) at –80°C prior to assay. Total protein values were measured using the method of Bradford (1976).

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Abbreviations

A	adenine
A	Ampere
AMV	Avian Myeloblastosis Virus
approx.	approximately
APS	ammonium persulfate
3-AT	3-amino-triazole
A. thaliana	*Arabidopsis thaliana*
A. tumefaciens	*Agrobacterium tumefaciens*
bidest.	double distilled water
bp	base pair(s)
BSA	bovine serum albumine
° C	degrees Celsius
C	cytosine
CaMV	Cauliflower Mosaic Virus
CAPS	3-cyclohexylamino-1-propane sulfonic acid
CIP	calf intestinal phosphatase
ColWT	wild-type *Arabidopsis* plants, ecotype Columbia
conc.	concentrated
cont'd.	continued
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
Fig.	Figure
G	guanine
g, mg, µg	gram, miligram, microgram
GluTR	glutamyl-tRNA reductase
GMP	P^1-5'-(7-methyl)-guanosine-P^3-5'-guanosine-triphosphate
GSA-AM	glutamate-1-semialdehyde-1,2-aminomutase
GUS	β-glucuronidase
h	hour(s)
IPTG	isopropyl-β-D-thiogalactopyranoside
KAc	potassium acetate
LiAC	lithium acetate
min	minute(s)
MS	Murashige-Skoog
4-MU	4-methyl-umbelliferone
MUG	4-methyl-umbelliferyl-β-D-glucuronide
NBT	nitro-blue tetrazolium
OD	optical density
o/n	over night
PCR	polymerase chain reaction
PEG	polyethylene glycol
PMSF	phenyl methyl sulfonyl fluoride
PP1	protein phosphatase 1
PP2A	protein phosphatase 2A

PVP	polyvinyl pyrrolidone
S. cerevisiae	*Saccheromyces cerevisiae*
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
Ser	serine
SSD	salmon sperm DNA
Strep	streptomycin
T	thymine
Tab.	table
T-DNA	transfer-DNA
TE	Tris-EDTA buffer
TEMED	N, N, N', N'-tetramethyl ethylenediamine
Thr	threonine
TRIS	trishydroxymethyl aminomethane
U	unit of enzyme activity
V	Volt
vol.	volume
v/v	volume per volume
W	Watt
WT	wild-type
w/v	weight per volume
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
X-Gluc	5-bromo-4-chloro-3-indolyl-β-D-glucuronide

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